OIL PALM FROND PETIOLE CONVERSION INTO BIOSUGARS AND BIOETHANOL

アブラヤシ葉柄の糖類とバイオエタノールへの変換

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May Allah bless all of us until the end. Ameen.

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LIST OF ABBREVIATION

AFOB	Asian Federation of Biotechnology
AIM	Agensi Inovasi Malaysia (Malaysian Innovation Agency)
APHA	American Public Health Association
ASM	Academy of Science Malaysia
BET	Brunauer-Emmet-Teller particle size distribution
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DTG	Differential Thermo Gravimetric
ETP	Economic Transformation Programme
FFB	Fresh Fruit Bunch
FPU	Filter Paper Unit
JSPS	Japanese Society for the Promotional of Sciences
HPLC	High performance liquid chromatography
NKEA	National Key Economic Area
OPEFB	Oil Palm Empty Fruit Bunch
OPF	Oil Palm Frond
OPMF	Oil Palm Mesocarp Fiber
OPT	Oil Palm Trunk
PKC	Palm Kernel Cake
POME	Palm Oil Mill Effluent
SHS	Superheated Steam
SEM	Scanning Electron Microscope
TG	Thermo Gravimetric

USDA	United States Department of Agriculture
WAXD	Wide Angle X-ray Diffraction
WDM	Wet Disc Milling
WWF	World Wild Life

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ABSTRACT

Palm oil industry is one of the Malaysian top commodities, producing abundant of lignocellulosic biomass as waste. Although a lot of studies have been done for these biomass, oil palm frond (OPF) has been neglected due to the current good agricultural practise, where it is required to be left in the oil palm plantation for nutrient recycling. However, a recent finding showed that the petiole parts of the OPF is rich in sugars, which can be extracted by pressing the petiole. As for the petiole biomass after pressing, it require pretreatment to allow enzymatic hydrolysis to take place smoothly. In the first chapter, a literature studies was carried out to understand the gap in the research for OPF, while trying to relate it with excessive energy of palm oil industry.

OPF from the oil palm plantation involves a volume of 83 million tons in Malaysia alone. As it was suggested earlier that the petiole alone is a good source for sugars, its utilization is restricted due to the good agricultural practise. Therefore, the second chapter discuss the comparison of oil palm biomass volume to justify the reason of OPF selection. Detailed composition of the OPF components was also studied to understand which parts of the fronds actually contribute to the nutrient recycling, as well as to suggest whether it is safe or not to take the petiole out of the plantation. Amongst the four components, namely petiole, stem, rachis and leaflet, we found that the main contributor for the nutrient recycling by OPF is actually the leaflet, while the petiole has only a little effect. We also suggest that the logistic issues regarding the transportation of the OPF petiole from the plantation to the palm oil mill, can be solved by adding extra cart behind the existing truck. Therefore we do not need extra truck and driver.

Third chapter is mainly about the pretreatment and cellulose hydrolysis of the petiole residue. We suggest that reuse of the oil palm steam (superheated steam, SHS) for pretreatment of petiole residue will be beneficial and profitable for the palm oil mill. The use of SHS is found to be superior as compared to a wet disc milling (WDM) in term of pretreatment duration and practicality in the palm oil mill. Upon treating the pressed petiole, we found that pretreatment of petiole at 180°C for 10 minutes yielded the highest sugars yield amongst the range of the treatment, which was achieved by using a low 10 FPU cellulase activity. This resulted in improvement of specific biosugars yield by 79.91% from untreated petiole residue. This short time pretreatment improvement is mainly due to the changes in specific surface area, particle size and properties of lignocellulose components which are discussed in detail throughout this study. We also suggest that pretreatment time also affected by residual oil such as found in oil palm empty fruit bunches (OPEFB) and oil palm mesocarp fibre (OPMF), therefore the petiole has advantage for not containing the oil residue.

In the fourth chapter, we study the effect of replacing the commercial nutrients for yeast by petiole's juice in bioethanol fermentation. This is due to a parallel study's finding which shows that the juice is rich in nutrients and some vitamins, which can support the yeast fermentation. Usage of petiole's juice to replace commercial nutrients in bioethanol production may reduce the production cost. We found that there was no significant difference in the fermentation performance when we replaced commercial medium with petiole juice. This concludes that the petiole juice is suitable as a supplement for bioethanol fermentation of OPF petiole hydrolysate.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Palm oil industry is amongst Malaysian biggest commodity in agriculture, which produces a huge volume of palm oil for domestic use, as well as for exports. It is an important industry in Malaysia, not only because it contributes to the national economy growth, it also provides the people with jobs and incomes. As the biggest agricultural business in Malaysia, it produces a staggering amount of solid and liquid biomass, both in the plantation, as well as in the mill. However the current palm oil mill in Malaysia still adapts technologies used decades back, which is low in efficiency of energy as well as lack in biomass reutilization.

Biomass from the palm oil industry actually has the potentials for the conversion to other value added products, through biotechnology and engineering approach. This should be applied to the most abundant biomass in the palm oil industry, which is the oil palm frond (OPF). Currently OPF is the least used biomass for conversion to other products. Meanwhile, energy such as steam which is being produced and used inefficiently in the palm oil mill, could be recycled for other purpose, such as for the biomass pretreatment and conversion means. Improving the steam energy efficiency by reutilizing it, as

well as making use of the wasted biomass, is important to enhance the palm oil industry perspective as the global clean industry. Furthermore, products produced from the oil palm biomass, such as biosugars, biofuel or biocomposite, would drive the industrial profit further, resulting to a higher economic value for the nation.

1.2 Objectives of the study

In the future, palm oil mill must produce lesser waste yet more energy and products to be sustainable. This involve usage of green energy and efficient in using it. With regards to the abundance of OPF produced in the industry, as well as enormous amount of steam wasted in the palm oil mill, it is important to adopt an efficient and sustainable technique to maximize the exploitation of these resources.

Hence, the objectives of this study are:

1. To investigate the properties of each components of the oil palm frond to understand its function and possibility of conversion into biomaterials and bioenergy,
2. To study the effect of SHS onto petiole residue as a pretreatment for the production of biosugars through enzymatic hydrolysis,
3. To produce bioethanol from sugars derived from petiole residue, with supplementation of nutrients from the petiole's juice.

1.3 Oil palm

Oil palm (*Elaies guineensis*) is an oil-producing plant which is native to west and southwest Africa, and commonly associated with massive scale production in Malaysia and Indonesia (USDA, 2007; USDA, 2012). Although it contribute up to 32% of global oils and fats, oil palm only use 5.5% of global land for cultivation among the 10 major oil seeds. Currently, Malaysia is the biggest global exporter of palm oil, amounting 44% of world palm oil, followed by Indonesia, although the latter has the largest planting area. As the second biggest commodity in Malaysia, palm oil contributed more than MYR80 billion in national export value (Table 1.1). Hence, the palm oil industry employs more than 600 000 workers, including high skills and low skills (Sime Darby, 2014).

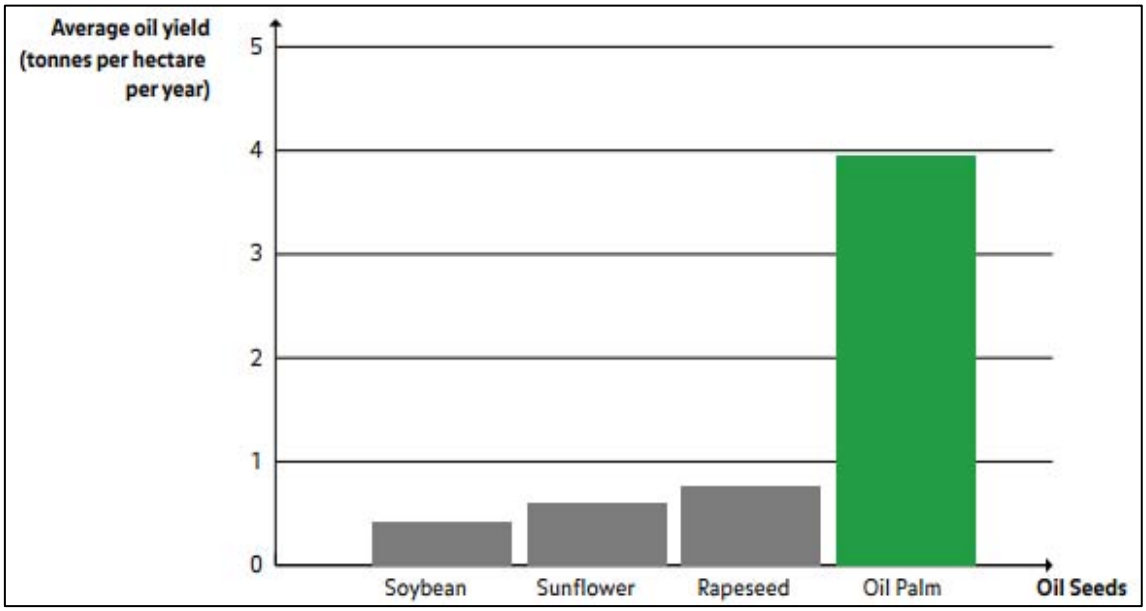
Table 1.1 Malaysian palm oil export value amongst the national commodities.

Year	Palm oil export value (MYR billion)	Export value of commodities (MYR billion)	Percentage of palm contribution in the overall export value
1980	2.89	48.80	6.1%
1990	5.50	20.70	26.6%
2000	14.94	42.72	35.0%
2007	44.71	88.70	50.4%
2008	65.22	112.43	58.0%
2009	49.59	91.2	54.0%
2010	59.79	113.3	52.8%
2011	80.30	130.0	61.8%

Source：Sime Darby (2014)

Oil palm is the most efficient oil-producing plant, yielding almost 4 tons per hectare per year, as compared to soybean, sunflower and rapeseed (Fig 1.1).

Furthermore, each oil palm tree can continuously produce fruits for about 25-30 years through it shelf-life, hence reducing the frequency to clear the land for replanting. The oil palms fruitlets grow together in a bunch, called fresh fruit bunch (FFB), with weight about 20-30 kg for each bunch. The FFB grows on at the base of the plant's leaf, which is called oil palm frond (OPF). Therefore in order to harvest the FFB, one or two OPF must be cut to allow the FFB to fall down after cutting. The OPF was then cut into smaller pieces prior to sort in a windrows form in the plantation, as a good agricultural practise to allow nutrients recycling (MPOB, 2014). The FFB on the other hand will be transported to the oil palm mill for oil extraction process.



(Figure adapted from Oil World, 2013; Sime Darby, 2014)

Fig 1.1 Oil yield from oil palm as compared to other oil-producing plants.

1.4 Oil palm biomass

Palm oil processing produces biomass including oil palm empty fruit bunches (OPEFB), oil palm mesocarp fibre (OPMF), palm oil mill effluent (POME), palm kernel cake (PKC), shells, oil palm frond (OPF) and palm oil mill effluent (POME) (Chew and Bhatia, 2008; Baharuddin *et al*., 2011). Meanwhile biomass such as oil palm trunk (OPT) will be produced at the end of the plant's life. The volumes of each indicated biomass are as shown in Table 1.2. Some of these biomass, for example OPEFB and OPMF, are being used in the plantation as a solid fuel to produce steam from river water, powering up turbine for electricity generation in the palm oil mill. Burning of the OPEFB often produce ash which is rich in potassium called bunch ash. OPMF and shells are also being sold to other companies for solid fuel purpose.

Table 1.2 Biomass produced throughout the palm oil mill processing.

Biomass	Volume (Million tons)
Empty fruit bunches	17.5
Mesocarp fibre	9.6
Shell	4.7
Frond	83.0
Trunk	15.2

Source : Sumathi *et al*., 2008

Although there are alternatives uses for some of the biomass, waste such as POME and POME sludge is regarded as a threat to the environment. Due to the high COD and BOD of the liquid (Wong *et al*., 2009), it is harmful to be released to the river even after pond treatment. However, studies are being carried out to

use such waste for biogas production in energy generation. On the other hand, biomass produces in the plantation such as OPF and OPT do not have any further usage for time being, except than being left in plantation for nutrient recycling (Abu Hassan *et al*., 1994).

1.5 Oil palm frond

In 2009, a total of 83 million tons of OPF was produced (ASM, 2010) and left in the plantation for nutrient recycling, as a good agricultural practise. It was stacked in a windrow form as a mulcher, while at the same time, slowly release the nutrients into the ground (Fig 1.2). However, previous study revealed that the oil palm frond (OPF) petiole contains a large amount of free sugars in moisture forms, which can be easily collected by just pressing it using a pressing machine (Zahari *et al*., 2012). Hence it is suggested that it is an excellent resource for the production of biosugars and bioenergy. In addition, Malaysian National Biomass Strategy (AIM, 2011) target efficient utilization of oil palm biomass mainly OPF for the production of value added products.

A

B

Fig 1.2 A typical OPF cut from oil palm tree (A) will be further cut into 3 or 4 pieces and (B) stacked in a windrow form in the plantation as a mulching as well as to assist nutrient recycling.

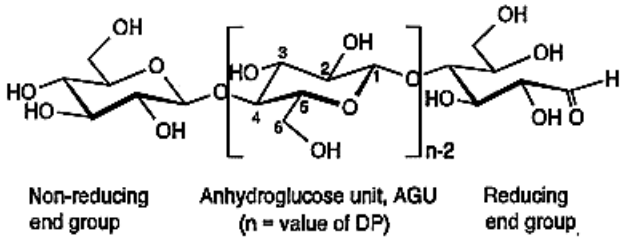
There is lack of information on which parts of the petiole actually contributes to the nutrient recycling. Since the report by Zahari *et al*. (2012), the composition of the OPF must be determined to understand whether usage of petiole outside of the oil palm plantation will cause disturbance to the nutrient balance or not. Additionally, similar characteristics of petiole shared amongst biomass is that it requires pretreatment to enhance the degradability by enzymatic hydrolysis. This is because a lignocellulosic biomass is built of mainly lignin, cellulose and

hemicellulose, in which cellulose and hemicellulose can be hydrolysed into its monomeric sugars.

1.6 Lignocellulose

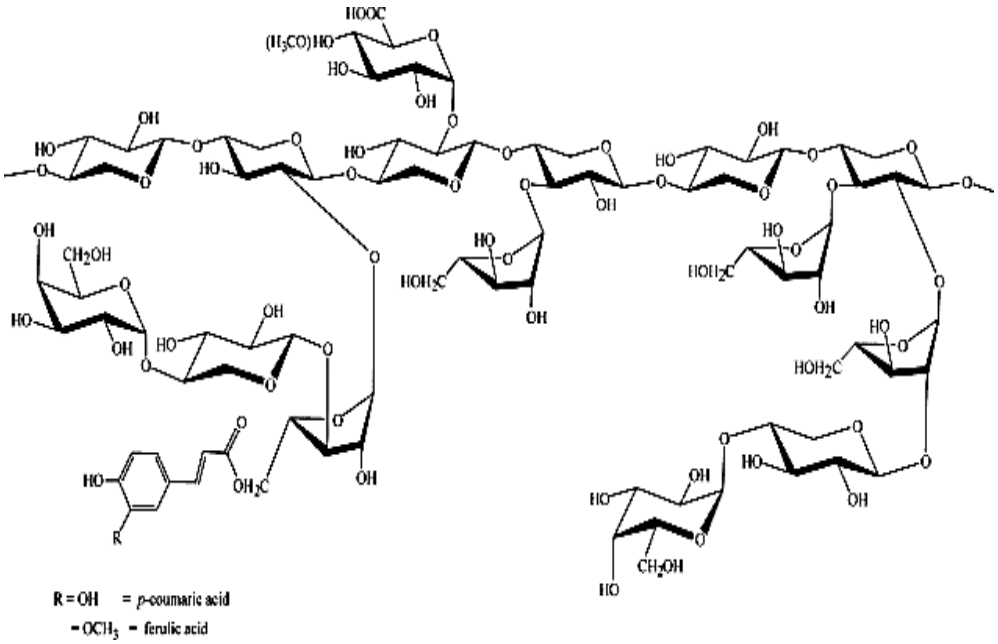
All biomass with major components of lignin, hemicellulose and cellulose (Fig 1.3) are commonly referred to as lignocellulose, or lignocellulosic biomass. This includes biomass from palm oil industry. Lignocellulose is the major parts building more than 60% of plant on earth (Tengerdy and Szakacs, 2003). Lignocellulose strength contributes by the complex organization between the three major components and their percentage. Basically, cellulose forms the structural skeleton and it is intertwine by hemicellulose which functions as matrix. Finally it is encrusted by lignin which forms the outer layer. Distribution percentages of each lignocellulose component generally distributed depends on plant species, age and environmental influence. Normally, lignin content of softwood will be much higher as compared to hardwood (Palmqvist and Hahn-Hägerdal, 2000).

A



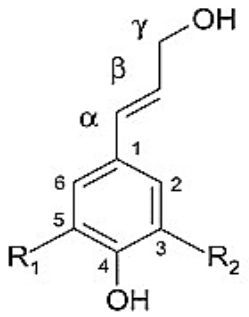
(Heinze and Liebert, 2001)

B



(Chaikumpollert *et al*., 2004)

C



1. $R_1=R_2=H$
2. $R_1=OCH_3$; $R_2=H$
3. $R_1=R_2=OCH_3$

(Buranov and Mazza, 2008)

Fig 1.3 Lignocellulose constituents. (A) cellulose, (B) hemicellulose, (C) lignin.

Cellulose is solely made of β-D-glucopyranose, or glucose, as its monomer (Fig 1.3A). Its strength is aided by a hydroxyl group exist in every cellulose chain, which form hydrogen bond with oxygen from the same or neighbouring chain. Depending on the locations of the hydrogen bonds, cellulose can be either crystalline or amorphous. This hydrogen bond also holds these chains together in side by side structure forming high tensile strength microfibrils. This microfibrils forms network in cell wall called carbohydrate matrix, which contribute to the rigidity of plant cells (Howland, 2000). However, cellulose strength and cyrstallinity can be reduced with the application of chemical, thermal or physical pretreatment (Hendriks and Zeeman, 2009). On the other hands, hemicellulose is made of many multiple sugar monomers such as glucose, mannose, galactose, rhamnose, arabinose with xylose as the major constituent (Fig 1.3B). Hemicellulose has shorter and branched polysaccharide chain, and embbedded in cell wall of plants where it cross-linked and chemically associated with microfibrils and lignin (Jeffries, 1990).

Lignin is totally a different complex chemical compound compared to cellulose and hemicellulose. With molecular formula of $C_9H_{10}O_2$, $C_{10}H_{12}O_3$, $C_{11}H_{14}O_4$ (Fig 1.3C), it is the most abundant biopolymer on earth after cellulose, contributing up to 30% of non-fossil organic carbon (Boerjan *et al*., 2003). Lignin content varied in different plant species, constituting from 10% until 33% of plants dried mass.While it is found naturally in higher percentage in compression wood as

compared to tension wood, it is one of the most slowly decomposing materials in a dead plant.

1.7 Palm oil mill

There are 421 oil palm mills (Fig 1.4) in Malaysia consist of government and private company producing 18.79 million tonnes of crude palm oil (CPO) in 2012. This industry is the biggest producer of solid and liquid agricultural biomass in Malaysia which are uniform and separated from each other. However, these palm oil mills are not very efficient in term of energy production and usage. Excessive energy is being produced and wasted, for example, biogas and steam (Yoshizaki *et al*., 2013). The biogas, mainly methane, was produced in the liquid waste treatment pond through methanogenesis by microorganisms due to high BOD, COD and other nutrients which support their growth.

(Figure adopted from www.salcra.gov.my)

Fig 1.4 A typical Malaysian palm oil mill during operation time.

River water which is being converted into steam (Ahmad *et al*., 2003) through the burning of OPMF, is used for the production of electricity by turbine (Lim *et al*., 2009). It is also being used for the pretreatment of the fresh fruit bunches, to separate the fruitlets from the bunch. However in current practise, it was estimated as much as 165 900 – 240 900 tonnes of steam is simply being released to the atmosphere (Yoshizaki *et al*., 2012), without tapping the energy which is still exist in the high temperature steam (Fig 1.5). This causes the mill's environment temperature increase. Moreover, in some mills, more OPEFB are being burnt just because it is too abundant and the mills need to get rid of such biomass. This may cause smoke and haze in the area which may lead to health hazard.

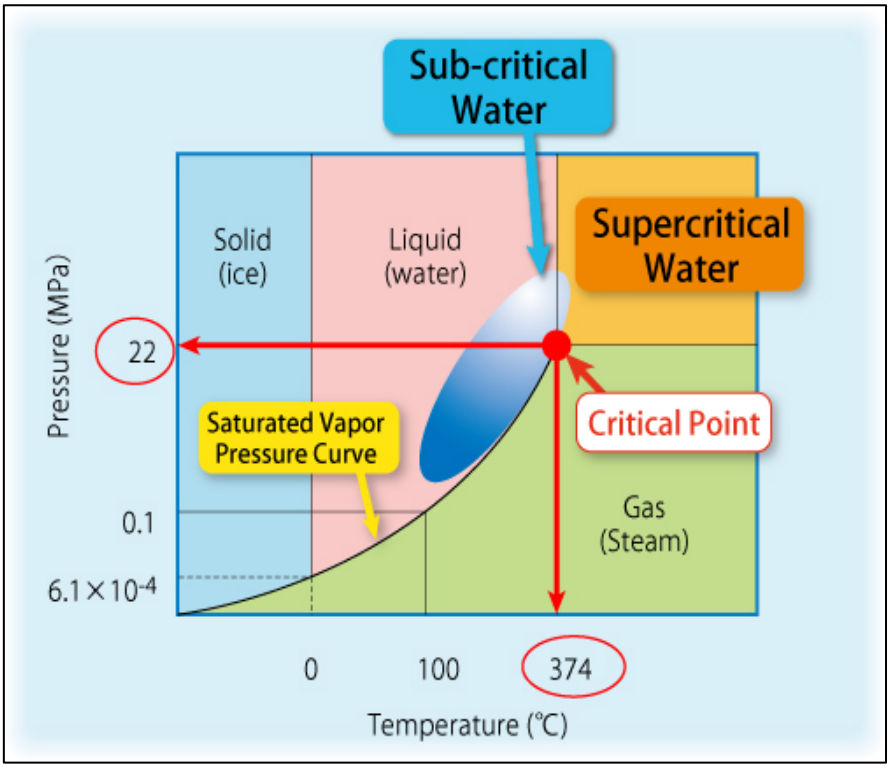
(Image courtesy of Kyutech, Japan)

Fig 1.5 Excessive steam produced by the palm oil mill is being released to the atmosphere.

1.8 Superheated steam (SHS)

The steam released from the palm oil mill can actually be characterized as a superheated steam (SHS). SHS is a sub-critical water condition at which dry steam is in gaseous forms which usually occur when a steam is being heated to a temperature between 100 to 374°C at atmospheric pressure (Fig 1.6). Therefore the steam can lose some of its internal energy while remaining in gaseous form (Liang *et al*., 2013). In most industry, SHS is being used for electric generation by turbine (Carapellucci and Giordano, 2013). This is

because the steam can flow through turbine passage as a compressible gas without damaging the moving parts of the turbine, which usually occur if there are liquid droplets such as in saturated steam.



(Figure adopted from: http://www.spiraxsarco.com)

Fig 1.6 The temperature and pressure where the superheated steam can form.

Studies suggest the use of SHS for drying of samples (Hasibuan and Wan Daud, 2009), but there are other studies related to its use for pretreatment of biomass (Bahrin *et al*., 2012; Nordin *et al*., 2013). In these studies, SHS was shown to have the capabilities to remove hemicellulose from the biomass due to the hemicellulose low degradation temperature as compared to cellulose and lignin. As a result, the biomass now loose its integrity as a solid lignocellulose complex, therefore in can be grind to produce a smaller particle as compared to

untreated. Nordin *et al*. (2013) also reported that by subjecting OPMF to SHS, lower degradation temperature parts will be removed, therefore, the biomass will become more stable thermally, suggesting that it will then be suitable for biocomposite.

1.9 Lignocellulose pretreatment

In order to convert a lignocellulose biomass into sugars, enzyme such as cellulase must be used to saccharify the cellulose into its monomeric sugars. However, pretreatment of the lignocellulosic biomass has to be carried out to improve the digestibility by enzyme. The pretreatment's objective is to simplify the lignocellulose structure, reduce the size of the lignocellulose as well removing parts which hinder the enzyme from attacking the cellulose (Sun and Cheng, 2002). Common technique used for pretreatment of lignocellulosic biomass includes the application of chemical, physical, biological, thermal including the application of high pressure, as well as combination of any pretreatment above (Hendriks and Zeeman, 2009).

Chemical treatment is the most common treatment as it is amongst the cheapest technique available. However it is disadvantageous because the chemical waste has to be treated prior to release to the mainstream (Iroba *et al*., 2013). Physical treatment on the other hand is quite an environmental friendly method, but the cost to build, run and maintain such equipment usually

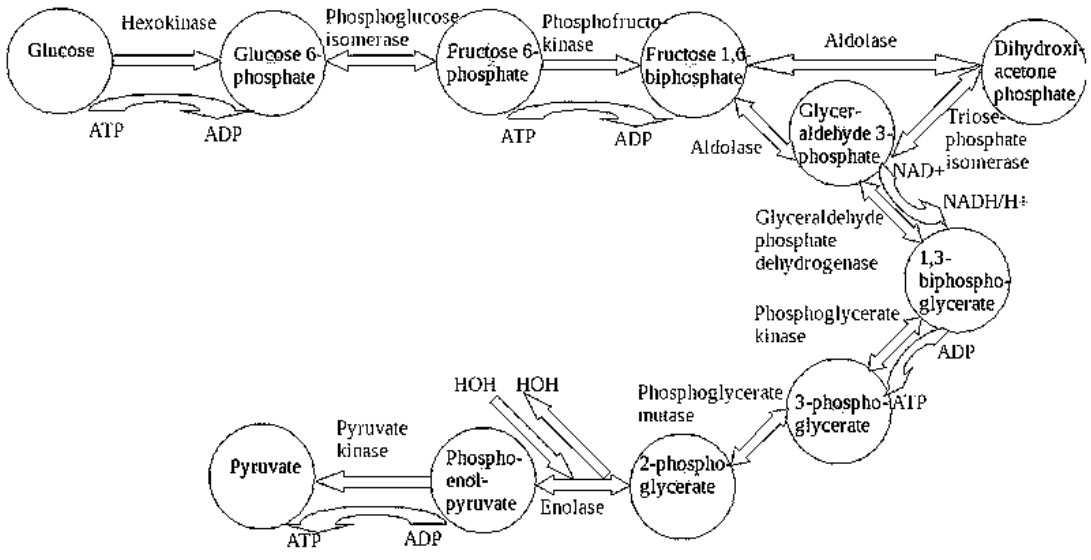
is high. The same is applied to thermal treatment, because to generate high temperature condition in a large scale usually involves a lot of energy (Acharjee *et al*., 2011). Meanwhile, biological treatment usually take long time as the growth of the microorganisms used must be taken into account (Wan and Li, 2012). Therefore most studies incorporate combination of those pretreatment to increase efficiency, as well as reduce pretreatment's duration.

1.10 Cellulase enzyme and saccharification

Cellulase enzyme functions as a catalyzer to enhance the degradation of cellulose into glucose, a process which is called cellulolysis, or saccharification (Singhania *et al*., 2010). Cellulase is built of three main components namely endo-cellulase, exo-cellulase and β-glucosidase. Endo-cellulase is an enzyme that cleaves both internal and exposed chain of cellulose, in both reducing-end as well as non-reducing-end chains, to produce cellobiosyl (Sukumaran *et al*., 2005). These shorter chain polysaccharide will then be digested by the exo-cellulase to produce cellobiose and cellotetrose, a two and four glucose molecules, respectively (Zhou *et al*., 2008). The cellobiose and cellotetrose will then be cleaved by β-glucosidase to produce a single monomer, glucose. The complexity of this process is the reason why high cellulase enzyme activity (measured in Filter Paper Unit, FPU), which is high in price, is required. This is also a factor of why an efficient pretreatment is required to reduce the concentration of cellulase enzyme used.

1.11 Bioethanol and ethanol fermentation

Bioethanol refers to ethanol which is being produced biologically commonly by yeast, as well as other acetone-butanol-ethanol (ABE) producing microorganism such as *Clostridium sp*., from organic sources such as agricultural waste or energy crop (Mola-Yudego and Aronsson, 2008). In current global trend, bioethanol fuel is being produced from sugarcane molasses. However traditionally, ethanol fermentation is being widely used around the globe in alcoholic beverages industry to produce *sake*, wine and other alcoholic drinks. This anaerobic fermentation involves the bioconversion of glucose into pyruvate through Embden-Mayerhof pathway, or simply known as glycolysis (Fig 1.7), and then the pyruvate will be converted into bioethanol, as in equation below:



(Figure adapted from Tortora *et al*., 2004)

Fig 1.7 Glucose conversion into pyruvate through glycolysis (Embden-Mayerhof pathway).

Bioethanol fermentation:

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

Usually the conversion will not reach a 100% due to the antimicrobial effect of high concentration of bioethanol. In batch fermentation, high concentration of ethanol will kill microorganisms, unless it is highly tolerant towards the solvent.

1.12 Lignocellulosic bioethanol as a sustainable bioenergy

In the renewable energy emergence, bioethanol is being produced as a fuel, either being use directly, partially or as an additive. In Brazil and United States for instance, bioethanol from sugarcane blend with gasoline, 20% and 85%, respectively, has been widely accepted to mitigate problems rose due to fossil fuel combustion and prices (Hall *et al*., 2009). Furthermore, a perfect combustion of bioethanol will only produce carbon dioxide and water such as in equation below:

$C_2H_5OH + 3\ O_2 \rightarrow 2\ CO_2 + 3\ H_2O$

Hence, bioethanol is considered as a clean, carbon-neutral fuel due to the fact that carbon dioxide that it produces is originated from plant's biomass, and it will be returned to plants again. It is also predicted that bioethanol is the fuel of the future, amongst biodiesel, biohydrogen and biogas.

CHAPTER 2: INVESTIGATION OF OIL PALM FROND PROPERTIES FOR USE AS BIOMATERIALS AND BIOENERGY

2.1 Introduction

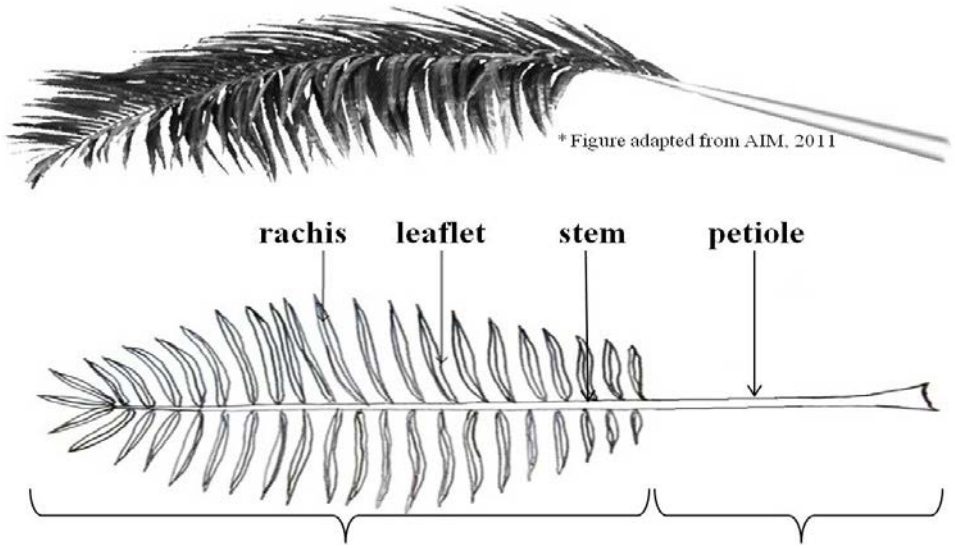
As mentioned earlier, OPF petiole has the potential for biosugar production, which can be obtained simply by pressing the petiole (Zahari *et al*., 2012). This finding is supported by the Malaysian National Biomass Strategy (AIM, 2011), to enhance utilization of oil palm biomass waste, increase productivity and profit of the oil palm mill, as well as generating new income for the nation as planned in Palm Oil National Key Economic Area (NKEA). However, current good agricultural practise suggest that OPF must be left in the oil palm plantation for nutrient recycling. Therefore few issues must be address in order to make use of the OPF petiole. Among them are (1) taking petiole out from the oil palm plantation will cause disturbance to the nutrient recycling, and, (2) transportation issue of the petiole from the plantation into the mill. This chapter will discuss our findings on the properties of each OPF components, which is the actual components that contribute to the nutrient recycling, and whether taking it out will affect nutrient recycling or not. Suggestion was also made on how it is possible to transport the petiole to the palm oil mill without the addition of extra truck or driver. We also compare the OPF petiole lignocellulose properties and production volume with other palm oil mill biomass which is readily available for

further exploitation, such as oil palm empty fruit bunch (OPEFB), oil palm mesocarp fibre (OPMF) and oil palm trunk (OPT) to explain why the use of OPF petiole is highly favourable in this study. Suggestion was also made on which biomass is more suitable as a non-food feedstock for biosugars production, as well as options of appropriate pretreatment for the selected biomass.

2.2 Materials and Methods

2.2.1 Samples preparation

OPF was collected from the oil palm plantation of University Putra Malaysia (Malaysia). It was separated into its components, which are leaflet, rachis (which held the leaflet), stem and petiole (frond part without leaves) (Fig 2.1). Stem and petiole were pressed using a sugarcane pressing machine to separate the petiole juice, prior to oven dried at 60°C for 48 hour, and subsequently ground using a hammer mill with 2 mm sieve. A higher yield of petiole juice during pressing can be obtained using a dual stage pressing machine (Mini Mill, Matsuo Co. Ltd) in Forest Research Institute Malaysia (FRIM) as shown in Fig 2.2. All samples, except petiole's juice were kept dry at 4°C until further used. Petiole's juice was kept at -4°C until further used.



Leaflet

Rachis

Petiole and stem

Petiole's juice

Petiole residue

Fig 2.1 Oil palm frond components.

Fig 2.2 Mini Mill (Matsuo Co. Ltd) used for pressing of OPF petiole to yield higher petiole's juice volume.

2.2.2 Thermo Gravimetric (TG) and Differential Thermo Gravimetric (DTG) analysis

TG and DTG analysis was performed using thermo gravimetric analyser (EXSTAR-TG/DTA7200 SII). Five to seven milligram of sample was subjected to heating from 50°C to 550°C and the weight differential versus time was measured. The remnant weight after the analysis was measured to determine the ash content.

2.2.3 Lignin, hemicellulose and cellulose content

Lignocellulose determination was performed according to the methods developed by Fahma *et al*. (2010). Extractives such as oil, fats and waxes were removed by Soxhlet extraction for 48 hours using an ethanol/benzene (1:2 v/v) mixed solvent. Lignin was then removed by soaking in (5% w/w) sodium chlorite ($NaClO_2$) solution (pH 4-5) for 1.5 hours at 70°C, then filtered and washed using deionised water. The residue was dried overnight at 70°C and the weight was measured (a). Hemicellulose was then extracted by soaking the residue after lignin removal in 6% (w/w) potassium hydroxide (KOH) for 24 hours at room temperature, then filtered and washed with deionised water (b). The residue was allowed to dry overnight at 70°C, prior to weight measurement (c). The lignin, hemicellulose and cellulose percentage then can be measured.

Initial lignocellulose - (a)	=	Lignin
(a) - (b)	=	Hemicellulose
(b)	=	Cellulose

2.2.4 Elemental and proximate analysis

Moisture content was calculated by drying the sample in a vacuum oven at 60°C for 48 hours. Elemental component (carbon, nitrogen, hydrogen and sulphur, CHNO) analysis was carried out according to standard methods. Nutrient and metal components were determined using x-ray fluorescent (XRF) equipment (Rigaku ZSX 101e). Crude protein was extracted and analysed

according to standard method (APHA, 1985). Pectin extraction was performed using method described by Yu and Sun (2013) while the starch content was measured by Humphreys and Kelly (1961) methods.

2.2.5 Total sugar analysis

Biomass hydrolysis by concentrated acid was performed according to the method of Sluiter *et al*. (2011). After neutralization, reducing sugars contents were measured by the DNS method (Wood and Bhat, 1988) while individual sugars was determined using a high performance liquid chromatography (HPLC) (Shimadzu LC-20A series) with Rezex RCM Monosaccharide Ca^{2+} (8%) column, installed with refractive index detector (RID) operated at 80°C, and distilled water as mobile phase at 0.6 ml/min.

2.3 Results and Discussion

2.3.1 Lignocellulose comparison between palm oil solid biomass: OPF, OPEFB, OPMF and OPT

Table 2.1 shows comparison between OPF, OPEFB, OPMF and OPT in term of volume production per year, as well as lignocellulose content. From this table, it can be clearly observed that the OPF is the main biomass produced from the palm oil industry (MPOC, 2010). Given that the OPF petiole alone weights half of the whole OPF, there is still as much as 41.5 million tonnes of petiole readily available for use every year, which is still higher than OPEFB, OPMF and OPT.

Furthermore, OPF petiole, which is currently not in use, also contain the highest concentration of holocellulose (combination of cellulose and hemicellulose), which is 84.56%, as compared to OPT (81.14%), OPEFB (79.46%) and finally OPMF (75.91). Holocellulose is the main components contribute to the sugars production during an enzymatic hydrolysis using cellulase enzyme. Therefore this shows the OPF petiole is a good candidate for the production of lignocellulosic sugars.

Table 2.1 Comparison in production volume and lignocellulose content of various biomass from palm oil industry.

Palm biomass	Units (million tonnes / year)*	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
OPF petiole	83.0	46.02	38.54	15.44	(This study)
OPEFB	17.5	51.22	28.24	15.19	Ariffin *et al*., (2008)
OPMF	11.3	42.81	33.10	20.49	Nordin *et al*., 2013
OPT	15.2	50.78	30.36	17.87	Lai and Idris, (2013)

* Value obtained from MPOC, (2010) for OPF, OPEFB, OPMF and OPT.

This data reveals that the OPF petiole is actually the most abundant biomass available in palm oil industry, which is actually very rich in holocellulose. Utilization of OPF petiole could make the palm oil industry advances one step further in green technology by conversion of OPF petiole into biosugars, as well

as other biomaterials such as biocompost, biocomposite and biofuel. Improving industrial capacity by utilizing biomass such as OPF petiole is a promising step to a better profit in the industry, generating jobs opportunities or peoples, as well as a promising greener environment. However, the properties of each OPF petiole components must first be determined to avoid disturbance in the nutrient recycling, as well as to choose the appropriate treatment, and maximizing the advantages of using it.

2.3.2 TG and DTG properties of OPF components

TG profiles of the rachis, leaflet and petiole are as shown in Fig 2.3. In this figure, the profile of the stem was omitted due to high similarity to the petiole. The profile of these 3 main components is widely spread from each other which show their materials durability against temperature. Petiole and stem, which can be considered as soft wood, is highly susceptible to thermal which will degrade the fibre structure quickly. This is mainly due to the arrangement of the fibre which make it porous and soft.

Leaflet on the other hand, started to degrade earlier and faster than petiole, with higher weight lost in the end. Rachis on the other hand, is built of dense and strong fibre materials to support the leaflet. This is why the remaining weight is very high. This suggest that during nutrient recycling in the plantation, leaflet will first degrade and release its nutrient into the soil, while being structurally support by rachis and stem for aeration and moisturising.

Although petiole shares the same properties of stem in TG, the absent of leaflet and rachis on the petiole means that it does not serve as structural support during the nutrient recycling process, except it will slowly degrade by itself. Hence, this encourages the studies of the nutrient content in all four components in the following step, in order to actually observe which component assist in the nutrient recycling.

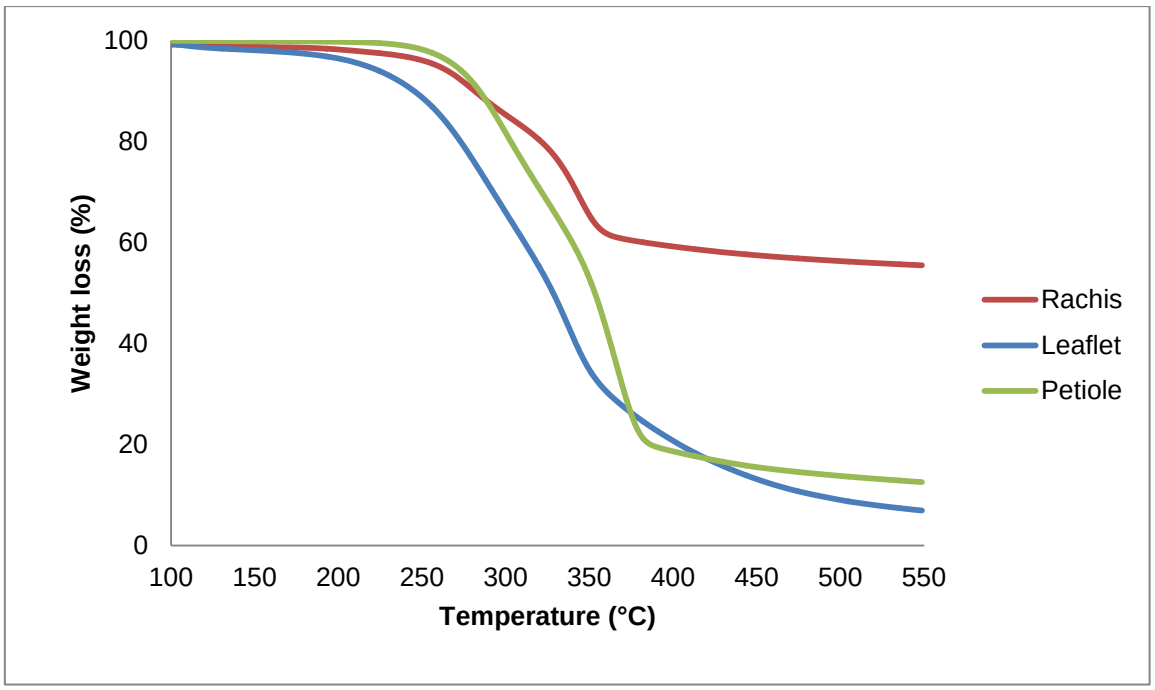


Fig 2.3 TG profiles of the rachis, leaflet and petiole of the OPF.

Meanwhile, DTG graph in Fig 2.4 show differences in materials that were lost at different temperature range. The initial observation made from this graph suggests that the rachis is highly rich in cellulose as compared to petiole and leaflet. Since the structure of rachis are compact and dense, the cellulose might be packed in a strong crystalline form, which could be one of the reason of why

rachis structure is tough and may remain the longest over time as compared to petiole, stem and leaflet. On the other hand, it can also be suggested that leaflet is richer in hemicellulose as compared to cellulose. This makes the leaflet softer in structure and will be the fastest biomass to degrade as compared to the other components. Meanwhile, stem and petiole, which shares the same properties in DTG, suggest a moderate amount of cellulose and hemicellulose content, whereby the cellulose concentration is much higher than that of leaflet. However, the actual lignocellulose will be confirmed in the following total composition analysis.

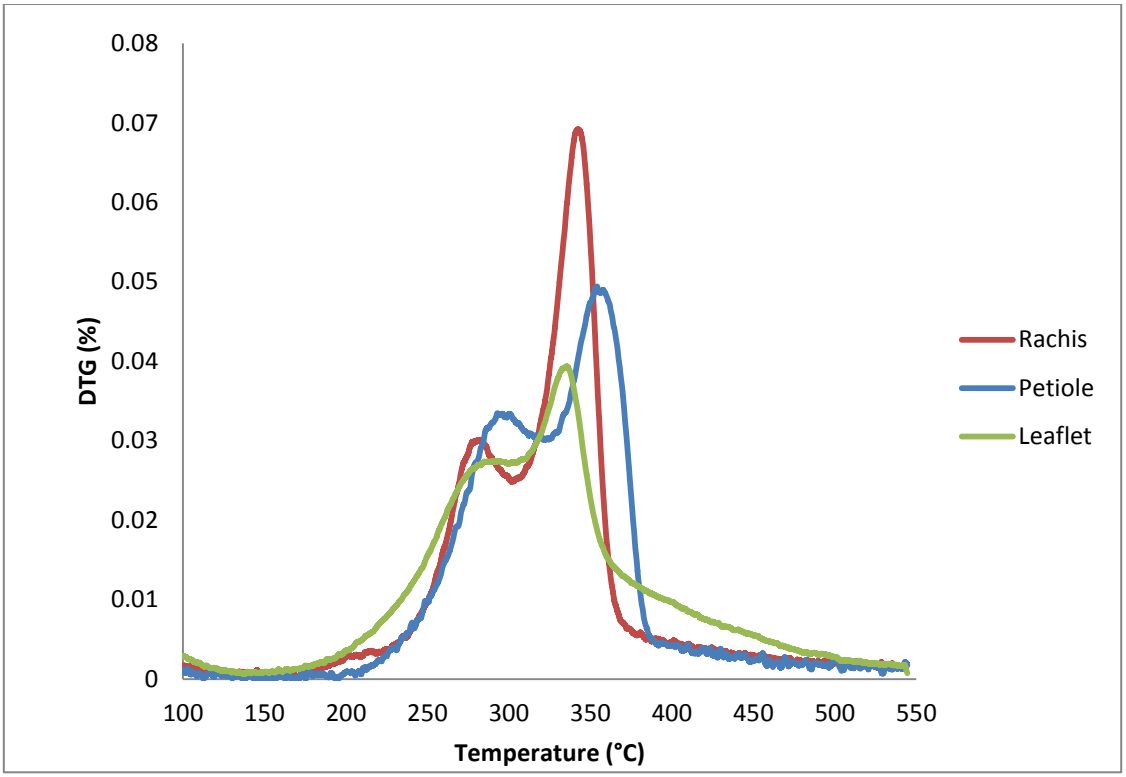


Fig 2.4 DTG profiles of the rachis, leaflet and petiole of the OPF.

2.3.3 Properties of OPF biomass components

OPF consists of four major components, namely petiole, stem, rachis and leaflet as shown in Fig 2.1. It was found that the petiole part accounted for half of the OPF weight (AIM, 2011). Observation of stem and petiole in SEM micrograph revealed the presence of porous lignocellulosic fibres with silica bodies, similar to those observed in OPEFB (Simarani *et al*., 2009). The moisture content of the OPF components varied, while C/N ratio of the leaflet, rachis, stems, and petiole was 25:1, 56:1, 90:1 and 77:1, respectively, as shown in Table 2.2. The concentration of pectin and protein of the petiole was almost comparable to that of the stem, but much lower than that of the rachis, while the leaflet showed the highest concentration of pectin and protein (Table 2.3). An inverse pattern was observed in the starch concentration which was much higher in petiole and stem, followed by the leaflet and rachis (Table 2.3).

Table 2.2 Carbon, hydrogen, nitrogen and oxygen (CHNO) analysis of OPF.

Analysis	Leaflet	Rachis	Stem	Petiole	Juice[3]
Hydrogen(%)	5.64	6.06	5.87	5.95	-
Oxygen (%)[1]	49.49	46.39	47.88	49.46	-
Carbon (%)	43.15	46.72	45.74	44.02	39.0
Nitrogen (%)	1.72	0.83	0.51	0.57	0.8
C/N ratio	25:1	56:1	90:1	77:1	49:1
Sulphur (%)[2]	ND	ND	ND	ND	0.4

[1]Oxygen content was determined by the difference between the contents of C, H, N and S in percentage and the total of 100%.
[2] ND – the sulphur content was below the detection limit of 2% of the method used.
[3] Data quoted from Zahari *et al*. (2012).

Table 2.3 Total composition of oil palm frond components.

Sample	Moisture	Percentage														
		Lignocellulose			Crude protein	Starch	Pectin	Elemental analysis								Total
		Lignin	Hemicellulose	Cellulose				Si	P	S	Cl	K	Ca	Mn	Others	
Leaflet	72.0 ±3.6	5.91 ±0.24	12.10 ±0.60	3.90 ±0.27	2.55 ±0.13	1.26 ±0.06	0.84 ±0.04	0.39 ±0.06	0.01 ±0.00	0.03 ±0.00	0.08 ±0.00	0.15 ±0.00	0.66 ±0.05	0.11 ±0.01	0.01 ±0.00	100
Rachis	60.0 ±2.1	1.79 ±0.11	13.85 ±0.55	19.57 ±0.97	1.76 ±0.09	0.94 ±0.06	0.24 ±0.01	0.26 ±0.02	0.02 ±0.01	0.04 ±0.00	0.12 ±0.04	0.43 ±0.07	0.87 ±0.01	0.10 ±0.00	0.01 ±0.00	100
Stem	75.0 ±5.3	2.53 ±0.12	7.42 ±0.51	11.41 ±0.45	0.80 ±0.05	1.55 ±0.07	0.06 ±0.00	0.08 ±0.02	0.01 ±0.00	0.02 ±0.00	0.08 ±0.00	0.49 ±0.03	0.55 ±0.00	ND	< 0.00 ±0.00	100
Petiole	77.0 ±5.4	2.86 ±0.11	7.15 ±0.35	8.53 ±0.59	0.90 ±0.04	1.87 ±0.09	0.07 ±0.01	0.15 ±0.03	0.02 ±0.00	0.03 ±0.00	0.11 ±0.01	0.50 ±0.00	0.77 ±0.04	ND	0.04 ±0.00	100
Juice[1]	-	-	-	-	NM[2]	-	NM[2]	-	0.02	0.40	-	2.30	2.90	2 ppm[3]	-	-

[1] Data quoted from Zahari *et al*., 2012
[2] NM – Data not mentioned
[3]Data are expressed in part per million (ppm)

It can be observed that cellulose is the major component of rachis, stem and petiole, while the leaflet consists of a higher concentration of hemicellulose and lignin (Table 2.3). As indicated in Table 2.2 and Table 2.3, leaflet shows the lowest C/N ratio which is suitable for use as fertilizer and soil conditioner. Gray and Biddleston, (1973) reported that a ratio below than 35:1 is required for microbial activity during composting, while a higher ratio results in slower composting rates. Therefore, based on the results obtained, the current practice for decomposing OPF in the plantation was adequate. Leaving leaflets in the plantation facilitates the return of nutrients to soil, while stem and rachis provide structural strength for aeration and space, as well as moisturizing during a raining condition. The structure of OPF itself is compatible with this practice except for the petiole.

As discussed earlier, petiole and stem have a high C/N ratio, which makes it not suitable to initiate composting process. However, stem is the part that provide structural support for the rachis and leaflet during nutrient recycling, which means that it is currently not available for further exploitation. Hence petiole, which does not play any role in structurally support the nutrient recycling process, is suggested as the part that does not belong to the nutrient recycling process. Since the petiole showed the highest moisture content of 77%, along with a lignocellulose content comparable to that of the other components, the capability of petiole bioconversion may improve after pressing for juice collection, which pretreats the petiole fibre.

The petiole mainly consists of cellulosic materials and sugars (Table 2.3). Therefore, its contribution to nutrient (nitrogen, phosphorus and potassium) recycling is low. The petiole only contributes up to 34% from OPF total nutrient content (AIM, 2011), with a comparatively low amount of nitrogen as compared to leaflet and rachis (Table 2.2). It was found that the main contributor for nitrogen is actually the leaflet (Tables 2.2 and 2.3), suggesting that the petiole could be used for other purposes, though its nutrients will not be recycled back to the plantation. Therefore, its role in the plantation should be investigated in another study in the future.

However, since the petiole can be supplied daily, it is a promising non-food feedstock for the production of bio-sugars and biomass raw materials. Furthermore, the petiole can be easily transported by the addition of another carriage to the same truck which brings the oil palm fruits to the mills (Fig 2.5). This simple solution solve the issues of cost, since no additional trucks and driver is required. Although a small cost might need to be added to provide the carriage. In addition, petiole volume collected during fresh fruit bunch (FFB) harvesting is much lower than the FFB, hence trips to send petiole is actually much lesser than the FFB.

(Figure edited from World Wild Life and Wikimedia, respectively)

Fig 2.5 Additional of extra carriage behind the existing truck to carry petiole can solve the issue of petiole's transportation.

However, there are also other issues that must be solved such as additional labour issue to handle the petiole parts, additional of cost for cellulase enzyme, as well as cost to build equipment and devices for these additional processes. Although labour issue could be minor, cellulase cost for biosugars production depends on the market. That is why pretreatment of the petiole residue must be carried out to reduce the use of the cellulase enzyme. On the other hand, building devices for the additional process may require some investment, but it will generate a better profit in the future. Hence, the company willing to involve in this process may have to first invest for the devices to achieve a higher profit later. Furthermore, applying this kind is technology in a palm oil mill is considered green and sustainable, which is a good advertisement for the company's professional image.

2.3.4 Potential of OPF for use for other value-added products

Petiole taken out from the plantation could be processed at the same palm oil mill due to the availability of excess energy in the production of palm oil (Yoshizaki *et al*., 2012, 2013). It was estimated that about 165 900 – 240 900 tonnes of superheated steam (SHS) is being produced in a palm oil mill, with about 50% of them is wasted due to inefficiency of the process, as well as released to the atmosphere after used without tapping the energy which is still trapped in it. This SHS could be used for thermal type pretreatment of the petiole after pressing, for the production of others bioproducts.

The SHS can also be used to dry the petiole residue. This allow the use of petiole residue as solid fuel source for the mill together with MF, or it can also be converted to biomass briquettes, and marketed as green energy. On the other hand, the stem which had been used as food pellet for cattle (Rahman *et al*., 2011) showed almost similar properties to those of the petiole in fibres and lignocellulose content similar to that of the petiole. Therefore, the petiole could replace the stem for bioconversion as food pellet. Hence, the stem together with the leaflet could still be used as fertilizer in the plantation.

Since it is difficult to obtain a high yield of OPF juice extraction during pressing, some sugars from juice will still be trapped in the petiole residue. It was experimentally confirmed, as shown in Fig 2.6, about one-third of glucose still remained in the petiole fibres. Saccharification should take place after juice

collection because by pressing, the petiole residue is physically pre-treated which facilitates its use. In the present study, 1 kg of OPF with 0.23 kg dry weight and 0.69 kg moisture content yielded a total of 152.42 g glucose, 59.97 g xylose, 13.34 g sucrose, 7.58 g arabinose and 1.09 g fructose, whereby more than 88% of hemicellulose consisted of neutral sugars (Fig 2.6). Sugars from saccharification and OPF juice can be used for bioconversion into bioethanol, bioplastics and other biopolymers, while biomass residue from these processes can be used as a source for biofertilizers as well as biocomposites.

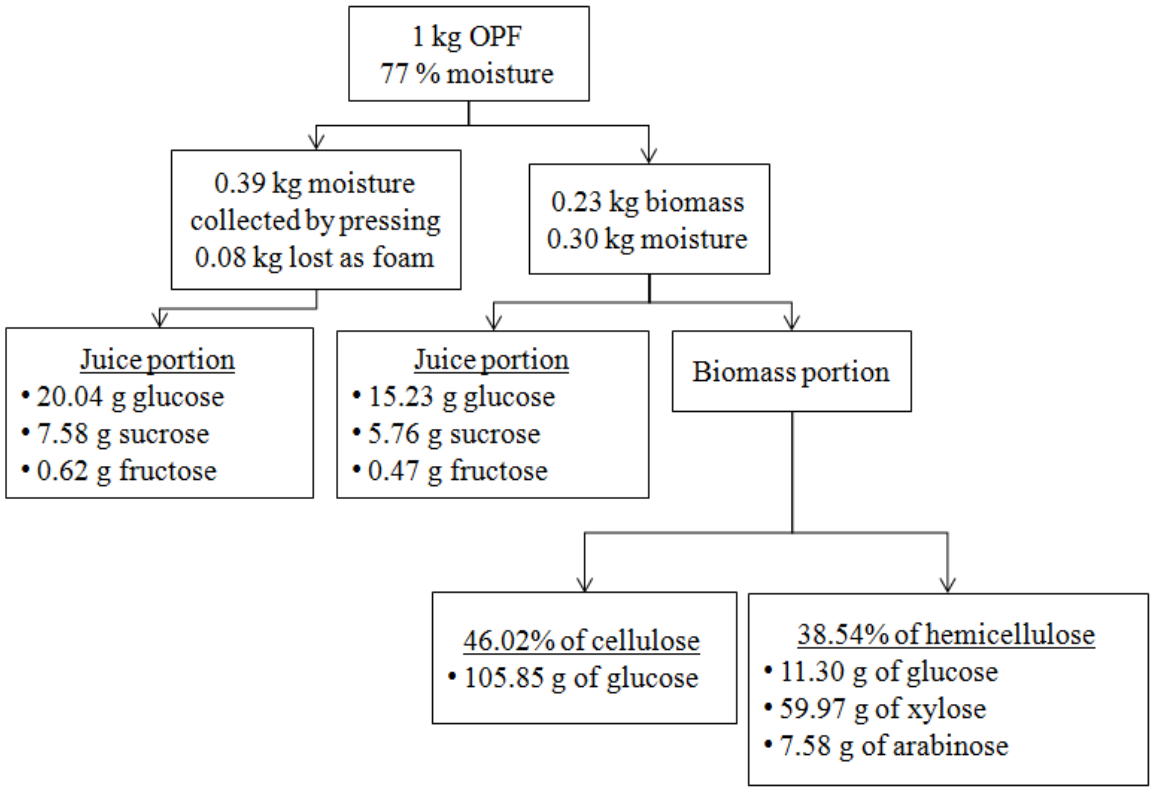


Fig 2.6 Theoretical sugars potential from OPF petiole.

In this case, if a sugar line production is to be introduced to a typical palm oil mill which receives about 45 tonne of FFB / hour, at maximum production, theoretical sugars production from OPF petiole will be as follow:

1 FFB (estimate 20 kg)	=	1 OPF petiole (estimate 3.5 kg)
45 tonne FFB / hour	=	7.875 tonne petiole / hour
7.875 tonne petiole / hour	=	1.846 tonne sugars / hour
		Whereby 1.2 tonne is glucose

This theoretical value could be varied in the real production line, but the value itself is promising. This is due to the facts that some of the sugars are readily available in the petiole biomass, which can be collected by just pressing, although by pressing alone will not yield as much sugars as from saccharification. This is the advantage key point of using OPF petiole as compared to other oil palm biomass such as OPEFB and OPMF, whereby petiole residue does not requires chemical pretreatment to produce sugars. OPEFB and OPMF on the other hand, are strong strand fibres which need extensive pretreatment in order to be used as lignocellulose source in an enzymatic hydrolysis process for sugars production (Bahrin *et al*., 2012; Nordin *et al*., 2013).

Apart from sugar line, petiole residue after pressing can also be converted to biocomposites because it contains lignocellulose with the presence of silica.

Neethirajan *et al*. (2009) suggested that the presence of silica bodies enhanced the structural strength of biomass while the addition of silica into composites can further strengthen its structure. Silica contributes up to 9.26% of petiole's metal content (Table 2.3) and all the nutrients still remain in the petiole biomass even after the juice is pressed out the juice. Therefore, this characteristic is suitable for the exploitation of the petiole for the production of a strong biocomposite. However, silica existence on the petiole's surface do affect the knives of the biomass choppers or grinder used during the biomass composite production, at which it will cause the knives to worn or erodes rather quicker than normal grinding of biomass with less silica content.

2.4 Conclusion

In conclusion, OPF's petiole shows a high potential as non-food feedstock for the production of renewable biosugars, biomaterials and bioenergy. Collection of only the petiole is advantageous because the juice and biomass can be utilized simultaneously, while the other parts of the OPF can still be left in the plantation for nutrient recycling to maintain the current good agricultural practice. These findings fully support the system, since it enables to generate more profit for business, as well as to achieve nutrient recycling for the plantation. However, additional studies related to the cost have to be conducted in the future to maximize the profit of this proposed process.

In the next chapter, the effect of SHS onto petiole residue will be studied and compare with other pretreatment method, in order to find an appropriate pretreatment method for the bioconversion of petiole residue into sugars. Different concentration of cellulase enzyme will be tested to find the optimum cellulase concentration to be used.

CHAPTER 3: SUPERHEATED STEAM AS AN APPROPRIATE PRETREATMENT TO IMPROVE FERMENTABLE BIOSUGARS YIELD FROM OIL PALM FROND PETIOLE RESIDUE

3.1 Introduction

In the previous chapter, it was shown that the main contributor of the nutrient recycling from oil palm frond is the leaflet, while rachis and stem provide structural supports for the aeration, so the leaflet can decompose easily. This allows the use of petiole for other purpose. It was also discussed that the palm oil mill generates excessive energy and steam which can be utilized for other purposes. In this chapter, the focus is on the superheated steam (SHS) generates by the palm oil mill, to use it as a means for pretreatment of the petiole residue after the juice was pressed out. The effect of a range of SHS temperature and duration on petiole residue characteristics was studied, and the pretreated petiole residue was subjected to hydrolysis by cellulase enzyme to study the response of enzyme on the treated petiole residue. The selected cellulase activity was used to demonstrate the effectiveness of SHS in degrading the petiole residue, because low cellulase activity used reflects to a low cost in the total sugar production, as enzymatic hydrolysis cost at this stage usually is the highest. Achieving a high sugars yield by SHS treatment means

that there is only a minimal cost needed for the pretreatment in the industrial scale, as the palm oil mill already producing it excessively. A comparison study using wet disc milling (WDM) as pretreatment were performed, as well as using different concentration of cellulase enzyme for saccharification, to see the efficiency of both parameters in the bioconversion of petiole residue. WDM was chosen as a comparison because it demonstrates a high yield of sugars after saccharification. However, the final verdict between SHS and WDM will be experimentally proved based on practicality for use in the palm oil mill.

3.2 Materials and Methods

3.2.1 Oil palm fronds (OPF) sample preparation

OPF were collected from oil palm plantation in Universiti Putra Malaysia. Rachis, leaflets and stem were removed by cutting to obtain the petiole alone. Petiole was subjected to compression by using a two-stage pressing machine (Mini Mill, Matsuo Co. Ltd) to isolate the biomass's moisture in juice form. The petiole residue which was considered physically pretreated during the process, was then oven dried at 50°C for overnight prior to grind using hammer mill with 2mm sieve. Ground petiole residue was kept in dried and ambient temperature.

3.2.2 Superheated steam (SHS) treatment condition

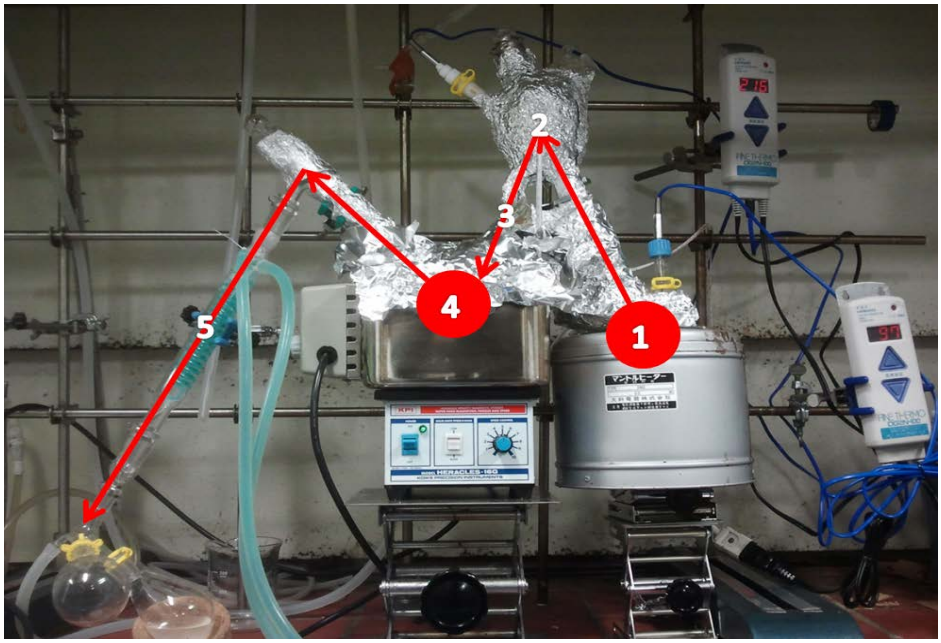
The temperature range and condition chose for this treatment was a modified condition based on a study on mesocarp fibre biocomposite (Nordin *et al*.,

2013) using a SHS generator with oven (Naomoto, Japan) (Fig 3.1A). The SHS conditions selected were 160°C, 180°C, 200°C and 220°C, each for 10 and 20 minutes. For each condition, the steam temperature was allowed to increase until the specific temperature prior to the loading of the petiole residue samples. After the retention time finished, petiole residue were quickly removed and cool down to room temperature to avoid any changes in the properties which is not caused by the SHS.

Lab scale superheated steam device was also developed to study oil extraction, as well as for material balance study of the SHS treatment condition of the oil palm biomass (Fig 3.1B). There are 5 stages in the device, which are steam generator, steam heater, temperature probe, treatment chamber, and distillation unit. Distiller unit utilize ethyl glycol as coolant, with set temperature of 5°C. Dried nitrogen was supplied into the collector unit after the distillation unit to compensate the pressure changes during the process, as well as avoiding moisture from external air to enter the chamber, which will disturb the material balance.

A

B



Lab scale SHS setup using laboratory equipment and glassware. (1) Heater to boil water and produce steam. (2) Heater to heat the steam up to SHS temperature. (3) Temperature sensor to read steam temperature prior to reach the biomass. (4) Chamber at which the SHS make contact with biomass. (5) Distillation unit to cool down the steam and collect any distillates. The system is fully closed and pressure changes inside the system are regulated with the supply of dried nitrogen to avoid external water vapour from entering the device.

Fig 3.1 Superheated steam machine used in this experiment: (A) SHS (Naomoto, Japan), (B) Lab scale SHS setup for material balance study.

3.2.3 Wet disc milling (WDM) pretreatment condition

In this pretreatment, petiole samples were treated using a wet disc miller (WDM) (AIST, Japan) (Fig 3.2) with the additional of water in a ratio of 1:10 (w/v). One hundred gram of petiole was soaked in 900 ml of distilled water prior to milling with a recycle number of 5, 10 and 20. A portion of the WDM-petiole residue was also treated by thermal at 121°C for 20 minutes to examine the effect of thermal onto the WDM products. All samples were then kept at 4°C until further used.

Fig 3.2 A wet disc miller (WDM) machine equipped with twin stone disc.

3.2.4 Particle size distribution, Brunauer-Emmett-Teller (BET) and surface area

All petiole residue samples were ground using a Warring grinder prior to sieving using autosieve machine with multiple metal siever for 10 minutes. Siever size in this study were 38 µm, 75 µm, 100 µm and 224 µm. BET surface area was calculated using Belsorp Adsorption/Desorption Data Analysis Software – Version 6.1.0.8.

3.2.5 Oil and chemical composition analysis

Oil extraction method was performed using Soxhlet extraction method using an ethanol/benzene (1:2 v/v) mixed solvent. Lignin, hemicellulose and cellulose analyses were done using method from Fahma *et al*., (2010). Lignin was removed by soaked in (5% w/w) sodium chlorite ($NaClO_2$) solution (pH 4-5) for 1.5 hours at 70°C, then filtered and washed using deionised water. The residue was dried overnight at 70°C and the weight was measured (a). Hemicellulose was then extracted by soaking the residue after lignin removal in 6% (w/w) potassium hydroxide (KOH) for 24 hours at room temperature, then filtered and washed with deionised water (b). The residue was allowed to dry overnight at 70°C, prior to weight measurement (c). The lignin, hemicellulose and cellulose percentage then can be measured.

Initial lignocellulose - (a)	=	Lignin
(a) - (b)	=	Hemicellulose
(b)	=	Cellulose

3.2.6 Thermo Gravimetric (TG) and Differential Thermo Gravimetric analysis (DTG)

TG and DTG analysis was carried out using EXSTAR-TG/DTA7200 SII analyzer. Five milligram of sample was subjected to heating from 50°C to 550°C and the weight differential versus time was measured. The remnant was weighed after the analysis to determine ash content.

3.2.7 SEM micrographs and surface roughness analysis

SEM micrograph was observed using Hitachi S3000N Scanning Electron Microscope. All pretreated petiole residue were coated with platinum with sputtering time of 30 seconds prior to observation under SEM. Petiole residue fibre's surface roughness measurement was performed using a computerized laser microscope (Keyence VK-X100).

3.2.8 Wide Angle X-ray Diffraction (WAXD) Analysis

Crystallinity of treated petiole residue was measured using Wide Angle X-ray Diffraction (WAXD, Rigaku Corporation) using Cu Kα as radiation source (λ = 0.154nm) at 40 kv and 50 mA with 2Θ range of 5° - 50°. The crystallinity index was calculated based on formula developed by Segal *et al*. (1959) as follows:

$$CrI = \frac{I_{002} - I_{am}}{I_{am}} \times 100\%$$

3.2.9 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was performed using an FTIR analyser, using potassium bromide (KBr) method. Petiole residue was pulverized using pestle and mortar, prior to mixing with powdered KBr in a ratio of 1:100. The mixture was then pressed to produce a translucent disc prior to viewing in the FTIR analyser. Spectrum was recorded in a wavelength ranged from 400 to 4000 cm^{-1}. The spectra were an average of 16 scans at a spectral resolution of 4 cm^{-1}.

3.2.10 Enzymatic hydrolysis for sugars production

Enzymatic hydrolysis was carried out using Acremonium cellulase (Meiji Seika) where 10 Filter Paper Unit (FPU) of cellulase activity was added for every one gram of petiole residue sample in a 30 ml of 0.05 M acetate buffer. In the comparison study, 50 FPU and 100 FPU of cellulase activity were added for every gram of petiole residue sample in a 30 ml of 0.05 M acetate buffer, respectively. Enzymatic hydrolysis was carried out at 50°C for 48 hours under sterile condition. Sugars were then analysed using HPLC and DNS method (Wood and Bhat, 1988). Unless stated otherwise, all experiments were done in triplicates.

3.3 Results and Discussions

3.3.1 Selection of an appropriate cellulase concentration level

Cellulase concentration level in the saccharification is important in determining the final product's cost. Although higher cellulase concentration used on biomass will produce higher sugar concentration, it will substantially increase the processing cost. Therefore the lower concentration of cellulase used is better to make it viable to industry, as well as affordable for the consumer. The selection of cellulase concentration range is based on other studies, which ranged from 10 – 100 FPU, in 48 hours (Jung *et al*., 2011; Jeon *et al*., 2014). Table 3.1 shows the effect of different cellulase concentration onto volume and yield of sugars produced, from untreated petiole residue.

Table 3.1 Comparison of different concentration of cellulase concentration onto the reducing sugars yield from untreated petiole residue.

10 FPU		50 FPU		100 FPU	
Specific sugar concentration (mg sugars/g treated biomass)	mg of sugars produced / 1 FPU	Specific sugar concentration (mg sugars/g treated biomass)	mg of sugars produced / 1 FPU	Specific sugar concentration (mg sugars/g treated biomass)	mg of sugars produced / 1 FPU
77.68	7.77	248.84	4.98	374.14	3.74

From Table 3.1 it can easily be observed that higher cellulase concentration produce higher concentration of sugars from SHS pretreated petiole residue. Cellulase concentration of 100 FPU exhibit highest sugars yield followed by 50 FPU, and finally 10 FPU. It is globally accepted that the usage of higher enzyme

will yield higher products, but the cost of the enzyme will be much higher, which will be reflected in the final cost of the products. Furthermore, higher enzyme concentration will not always be as efficient as lower enzyme concentration.

Careful observation reveals that, using 10 FPU on untreated petiole residue will yield 7.77 mg of sugars per FPU, followed by 50 FPU and 100 FPU which is at 4.98 mg sugar / FPU and 3.74 mg sugars / FPU, respectively (Table 3.1). This shows an efficiency reduction by more than 50% with the use of 100 FPU as compared to 10 FPU. However, higher volume of petiole residue will be used in the 10 FPU condition as compared to 100 FPU, but currently this is not the main concern. This is because, there will be a huge amount of petiole residue produced from the pressing of petiole to extract the petiole juice, at which the palm oil mill might not be able to utilize all of the petiole residue for sugars production, due to limitation of the steam.

With the use of 10 FPU, there will be wet petiole residue waste after the saccharification which is still rich in lignocellulose. It is considered as a good candidate for another bioconversion such as for biocompost or as a mulcher, or it can also be further treated using SHS again for the production of biocomposites. Hence following this study, a concentration of 10 FPU was selected for subsequent saccharification.

3.3.2 Saccharification of SHS treated petiole residue

Saccharification was carried out to measure the effect of SHS treatment on petiole residue in enzymatic hydrolysis. From Fig 3.3A and 3.3B, sample undergo SHS at temperature 180°C for 10 minutes exhibit highest sugar production amongst the other samples, followed by treatment at 160°C and 200°C. These samples produced sugars much higher than untreated petiole residue, while treatment at 220°C produced slightly less. However actual performance of the SHS can only be seen from Table 3.2 where the specific sugars yield after the weight lost during SHS was calculated. It is worth to note that sugars yield do increase as much as 79.91% for petiole residue undergoing SHS treatment at 180°C for 10 minutes as compared to untreated. This result is in accordance to lignocellulose data from Table 3.3 whereby the sample has the highest concentration of cellulose as compared to other treatment.

On the other hand, treatment at 220°C for 20 minutes appears to produce lower sugars as compared to untreated. This might be due to the crystallinity of the fibre which increases in parallel to the treatment temperature and time. Similarly it was also reported that SHS treatment at more than 200°C for 60 minutes caused partial cellulose degradation leading to lower sugar yield (Bahrin *et al*., 2012), although in this study petiole residue becomes blackened even after 10 minutes at 200°C indicating charring has started to occur. At the same time cellulose concentration also started to reduce with dramatic increment in lignin concentration which leads to the reduction in saccharification.

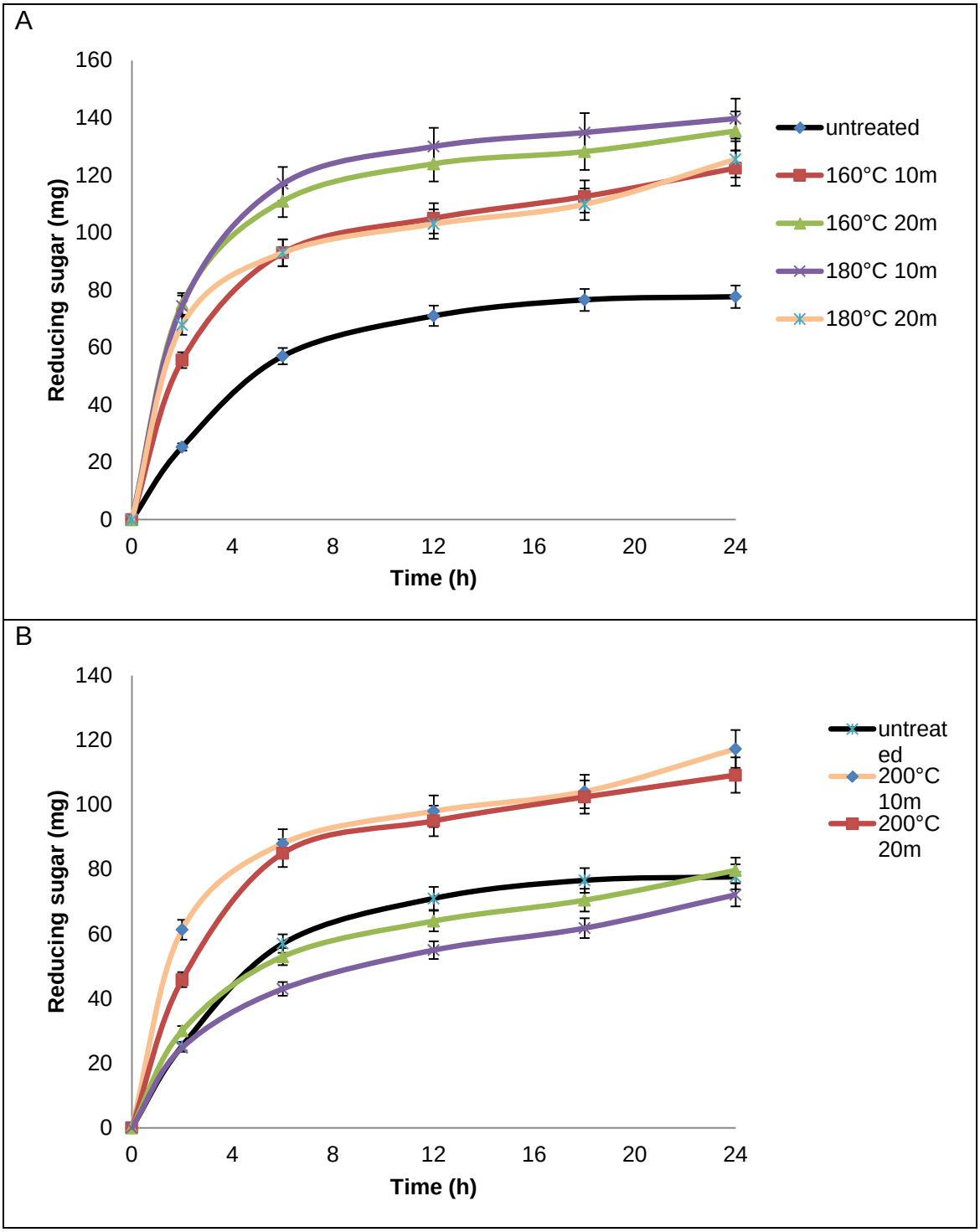


Fig 3.3 Reducing sugars concentration profile during saccharification of (A) untreated sample and treated sample temperature in the range of 160°C - 180°C; (B) untreated sample and treated sample in the range of 200°C – 220°C.

Table 3.2 Performance of SHS treated biomass. Specific sugars yield based on weight of petiole residue.

Sample	Percentage of weight lost (%)	Specific reducing sugars (mg sugars/g treated biomass)	Sugars yield (g sugars/g of petiole)	Increment in specific sugar yield (%)
untreated	0.00	77.68	77.68	0.00
160°C 10m	15.70	122.48	103.25	57.68
160°C 20m	17.00	135.43	112.41	74.35
180°C 10m	16.60	139.75	116.55	79.91
180°C 20m	18.20	125.54	102.69	61.62
200°C 10m	17.50	117.27	96.74	50.97
200°C 20m	19.60	109.17	87.77	40.55
220°C 10m	22.50	79.68	61.75	2.58
220°C 20m	26.10	72.12	53.30	-7.15

3.3.3 TG and DTG analysis

In this study, a range of superheated steam conditions was applied as additional pretreatment for the petiole residue. Short time exposure to SHS appears to have substantial effect on the lignocellulose components which are translated into the DTG and TG profiles. DTG profiles of samples (Fig 3.4A and 3.4B) suggest the increment in peak of the cellulose region, which at the same time, are shifted to a higher temperature region, as the treatment temperature and time increases. Hemicellulose shoulder reduction was also observed with increment of time mainly due to depolymerisation of xylan chain (Bahrin *et al*., 2012).

The reason why the SHS effect is evident on the petiole residue is due to the pressing step for juice extraction. Pressing step pretreats the petiole residue

physically, whereby it was crushed into uniformed fibrous, while at the same time exposing the internal parts. SHS also have additional advantages for pressed petiole. For example, the SHS treated petiole residue can now be subjected to other pretreatment which is not suitable in the initial form, which requires smaller size fibre for substantial effect. SHS also caused the petiole residue to dry, which helps if the biomass need to be transported from the mill. This pretreatment can also reduce the risk of contamination by fungus during petiole storage, which is a common problem with the storage of OPEFB.

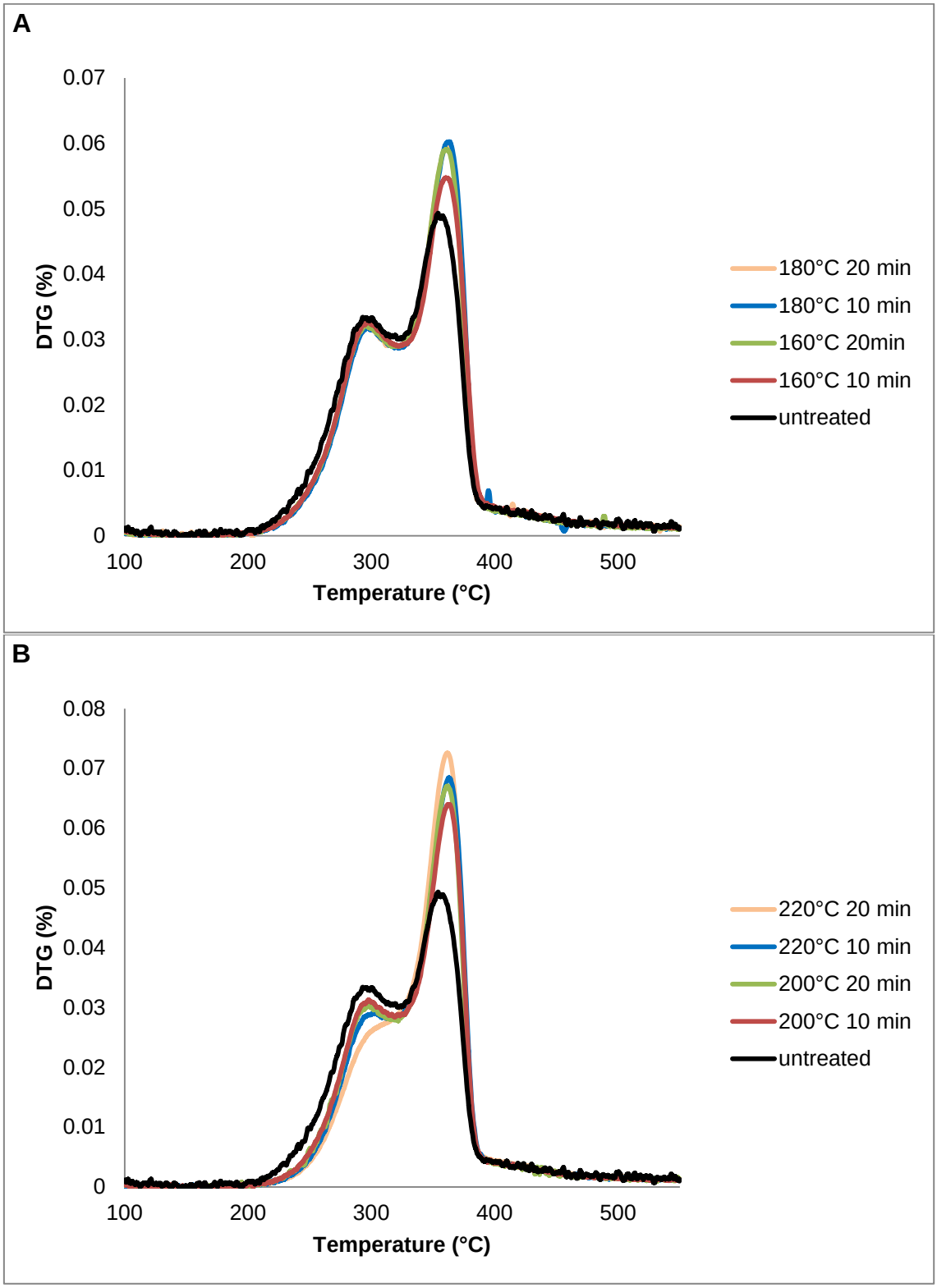


Fig 3.4 Comparison of DTG of petiole residue after various conditions of SHS. A) comparison of untreated with 160°C - 180°C, B) comparison of untreated with 200°C - 220°C.

TG result of petiole residue after various condition of SHS treatment (Fig 3.5) shows that after treatment, the petiole residue profiles shifted to higher temperature range with less residual weight at higher treatment temperature and prolonged treatment time. This is due to the partial removal of components with lower degradation temperature such as hemicellulose and other impurities. This finding is in accordance with studies using other parts of oil palm such as OPEFB and OPMF which were achieved at longer treatment's period (Bahrin *et al*., 2012; Nordin *et al*., 2013). This suggests that the petiole residue acquired substantial thermal stability after the treatment, partly due to the removal of some of hemicellulose but maintaining the lignin (Sagehashi *et al*., 2006).

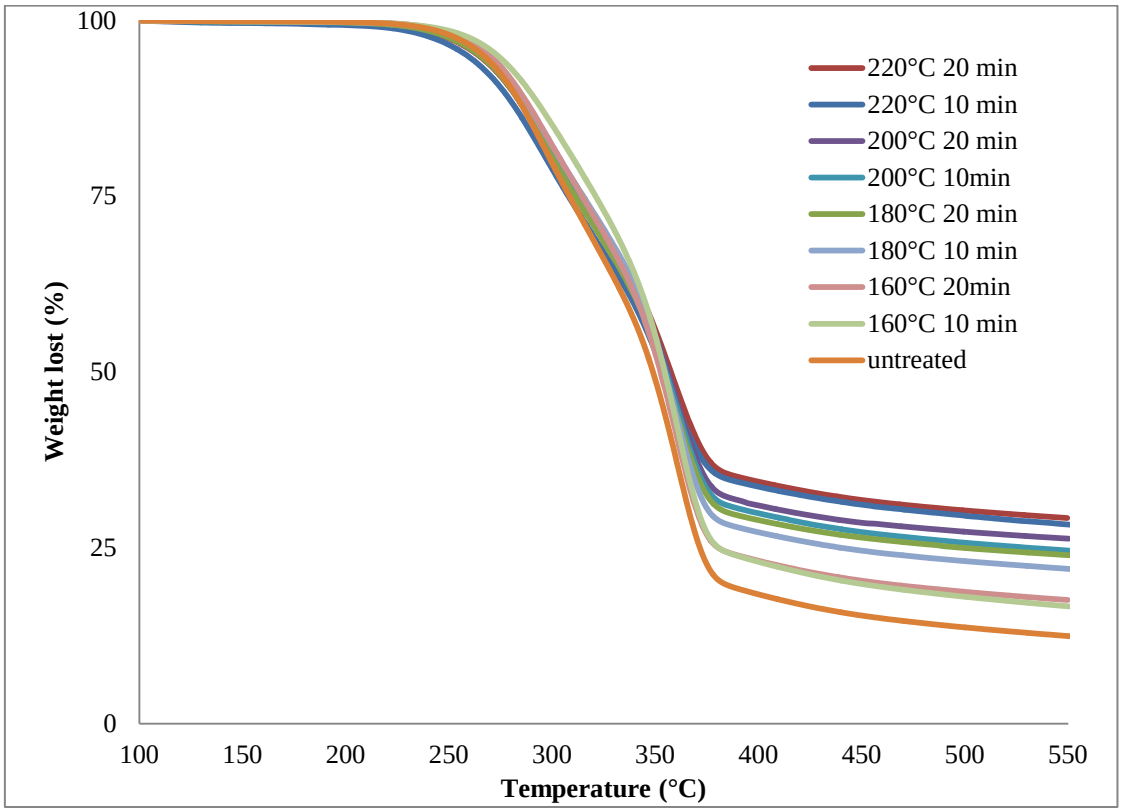


Fig 3.5 TG profiles of petiole residue undergoing various condition of SHS.

3.3.4 Effect of SHS on lignocellulose components of petiole residue

Percentage of lignocellulose components and weight loss of petiole residue after SHS treatment are shown in Table 3.3. These results responded very well with findings on TG and DTG whereby from the starting treatment temperature of 160°C, considerable amount of hemicellulose was removed, and keep reducing gradually until 220°C. This distinctive property of hemicellulose is contributed by soft polyoses chain and branch, which is susceptible to thermal treatment (Sagehashi *et al*., 2006; Hendriks and Zeeman, 2009).

On the other hand, cellulose, which is built of straight chain polymer with hydrogen bond linking those chains, is more resistant to thermal treatment as compared to hemicellulose. Although studies reveals that cellulose degradation temperature is in the range of 338-354°C, it can be reduced to 192-238°C by the addition of $ZnCl_2$ (Amarasekara and Ebede, 2009). The percentage of cellulose from petiole residue in this study peaked at treatment condition of 180°C for 10 minutes and started to drop afterwards. This finding is corresponded with the data on sugars yield where the highest sugars yield came from sample treated with 180°C SHS for 10 minutes.

Table 3.3 Effect of SHS onto lignocellulose content of petiole residue. Lignocellulose content after saccharification is shown to compare the degradation of the lignocellulose after the process.

	Lignocellulose content of SHS petiole prior to saccharification				Lignocellulose content of SHS biomass after saccharification	
	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Weight lost during treatment (%)	Cellulose (%)	Hemicellulose (%)
Untreated	46.02	38.54	15.44	0.00	30.87	38.15
160°C 10m	55.02	18.01	26.97	15.70	31.14	17.40
160°C 20m	54.79	17.06	28.15	17.00	28.38	16.38
180°C 10m	56.56	14.99	28.45	16.60	29.31	14.29
180°C 20m	56.33	13.34	30.33	18.20	31.85	12.71
200°C 10m	54.84	13.17	31.99	17.50	31.97	12.58
200°C 20m	52.11	12.91	34.98	19.60	30.82	12.36
220°C 10m	50.65	12.08	37.27	22.50	35.11	11.68
220°C 20m	49.52	10.45	40.03	26.10	35.46	10.09

The reason why hemicellulose and cellulose from petiole residue is more prone to the SHS treatment could be due to the pressing for juice collection, which highly destroys the fibrous strength, and producing powdered and dusty fibre after grind, which has higher surface area. This suggests that SHS make more contact with the petiole residue fibre, which facilitates the hemicellulose degradation process. Hemicellulose removal allow cellulase enzyme to have better access to cellulose leadings to better sugars yield.

Lignin on the other hands differs from both hemicellulose and cellulose by keep increasing in percentage all the way across the temperature and time ranges. This is because lignin is built of strong aromatic groups and is highly branched which contribute to its structural strength. However, considerable damage to the biomass also occurred whereby the weight lost becomes more evident at higher temperature, due to the hemicellulose decomposition, silica body removal and volatization of some components (CO, CO_2, CH_4 and some hydrocarbons) (Yang *et al*., 2007). Therefore suitable temperature can be chosen to find a good balance between cellulose and lignin increment, hemicellulose decrement and weight lost.

3.3.5 SEM micrograph

Phytoliths (silica bodies) can be easily found on all untreated oil palm biomass such as on OPEFB, OPMF as well as OPF (Baharuddin *et al*., 2011; Chua *et al*., 2009; Roslan *et al*., 2014). In this study, silica bodies can also be seen

throughout the petiole residue samples (Fig 3.6). It was reported that silica bodies could be one of the hindrance for optimum saccharification (Neethirajan *et al*., 2009) therefore its removal could help to increase the hydrolysis rate due to increase in porosity and surface area. Bahrin *et al*. (2012) found that 60 minutes of SHS treatment on OPEFB at 180°C partially removed the silica bodies while 210°C almost fully removed them all, although the 180°C treatment with less silica bodies removal yielded highest sugar yield. The finding was also in accordance to Nordin *et al*. (2013) where the treatment were in the range of 210°C to 230°C from 1 hour to 3 hours to remove silica bodies on OPMF.

However in this study, shorter time of treatment is the main objective (10 – 20 minutes). Therefore silica bodies removal is not apparent at lower treatment's temperature although it can be seen that longer treatment have more effect on the silica bodies at the same temperature. Silica bodies removal becomes more evident at 220°C at both duration of 10 and 20 minutes. Although longer duration of treatment may remove more silica bodies, it is physically clear that the short duration SHS affects the petiole residue's fibre. This is based on the colour and texture of the fibre after the treatment which becomes finer, blackened, more uniform in size and brittle. Small and fine fibre may results in a higher degree of enzymatic hydrolysis, yielding more sugars, although until a certain point, other factor such as crystallinity may become a hindrance (Ogeda *et al*., 2012).

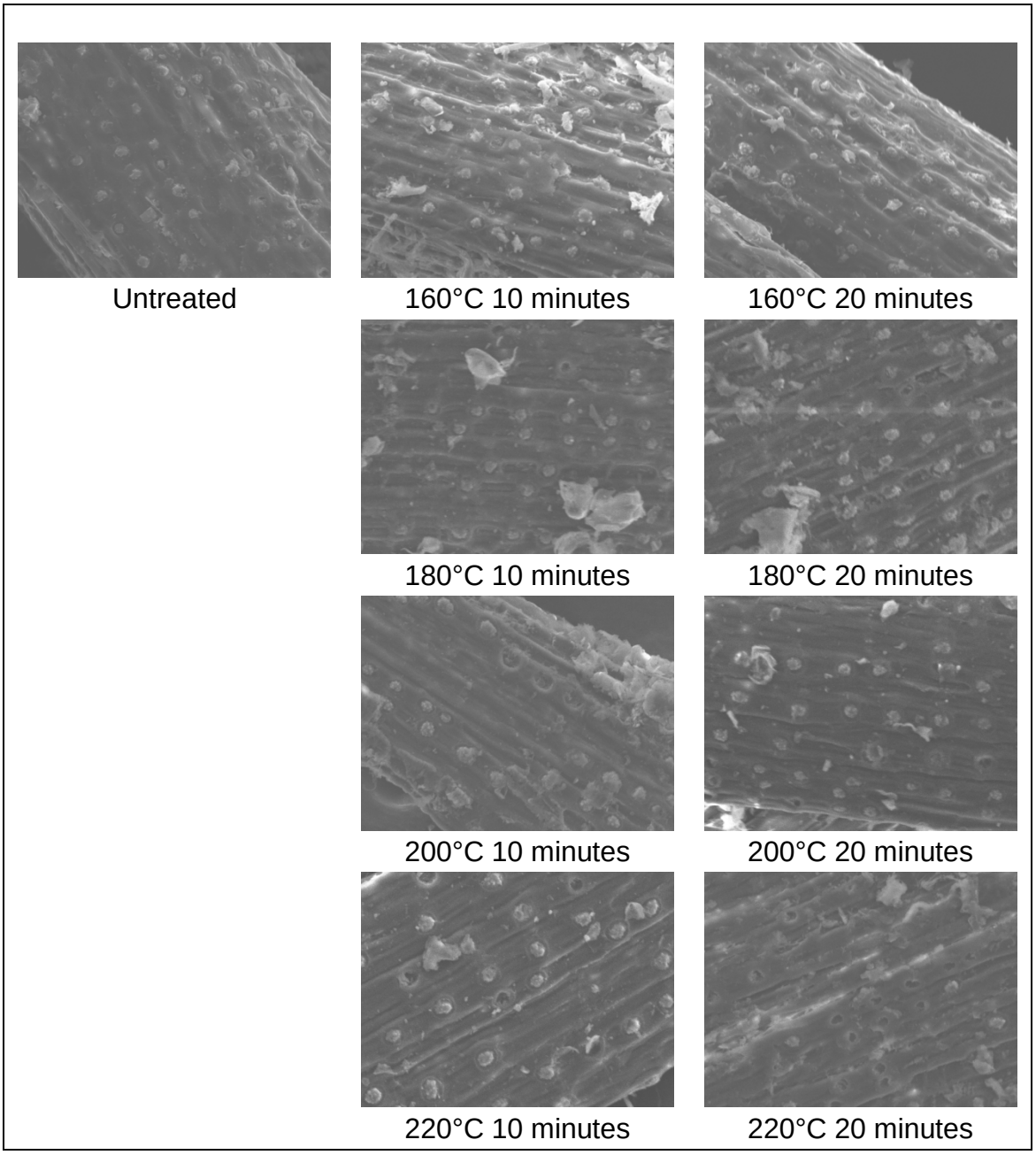


Fig 3.6 SEM micrograph showing silica bodies on the petiole fibre prior to and after SHS treatment.

3.3.6 Wide Angle X-ray Diffraction (WAXD) Analysis

Fig 3.7 shows X-ray diffraction pattern at 2Θ for the cellulose from petiole residue. It was calculated that the untreated petiole residue have a crystallinity

index of 40.7% which immediately dropped during pretreatment between 160°C 10 minutes to 180°C 10 minutes. Afterwards, the crystallinity index gradually increases with prolonged pretreatment time and temperature whereby at 220°C 20 minutes treatment condition, it surpasses the crystallinity index of untreated petiole residue. It was suggested that during lower temperature pretreatment, some of the impurities was removed, hence reducing the crystallinity index of the untreated petiole residue. This is supported by another finding (Bhuiyan and Hirai, 2000) where it was found that other components inside wood cellulose are involved in increasing the crystallinity, hence the removal of such components will result in reduction in crystallinity.

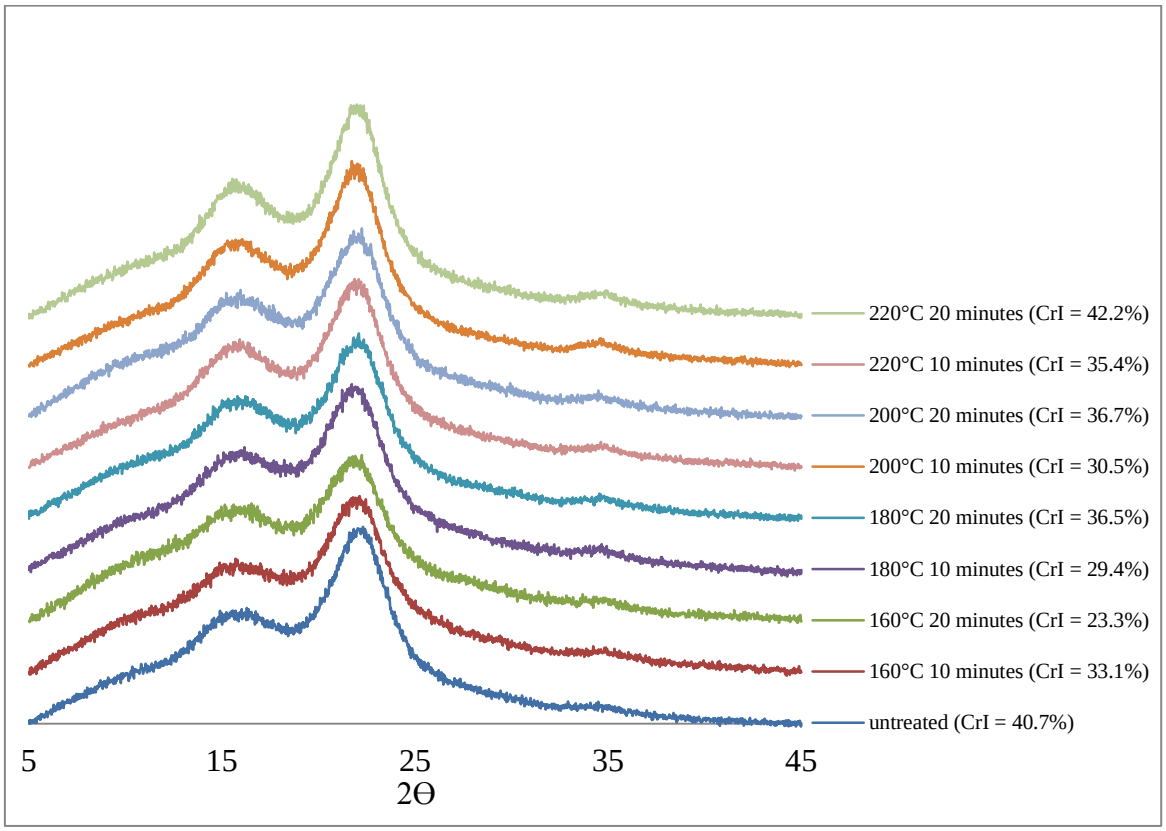


Fig 3.7 X-ray diffraction pattern of petiole residue pretreated with SHS at different condition.

Meanwhile, higher temperature and longer period allow the biomass to become a fibre with higher degree of crystallinity, although at higher temperature and longer duration, the fibre structure will be disrupted, leading to a much lower crystallinity index (Nordin *et al*., 2013). This data, combined with the lignocellulose content data (Table 3.3), is an evident of why the 180°C for 10 minutes condition is by far the most suitable pretreatment of petiole residue by SHS. The crystallinity of petiole residue, which is a soft fibre, seems to be highly affected while at the same time, still consist of high cellulose content. All of this effect combined lead to a higher saccharification degree by cellulase enzyme.

3.3.7 Particle size distribution, BET surface area

Due to the removal of hemicellulose and moisture after SHS, the petiole residue now becomes brittle and easier to grind, producing finer particles. According to Fig 3.8, particle sized more than 224 µm generally shows gradual reduction in percentage from 160 – 200°C and sharply reduce at higher than 200°C. On the other hand, particle sized between 224 – 100 µm and below 100 µm is gradually increase with the increment in temperature and time. This result is in accordance with the increment in specific surface area data in Fig 3.9.

The size of biomass's particles and porosity is important in enzymatic hydrolysis since particle with smaller size and more porous have larger surface area, which allow higher degree of hydrolysis (Palonen *et al*., 2004). Smaller biomass particle size also has advantage in reducing the enzymatic hydrolysis cost. This

is due to the higher surface area of smaller biomass particle require lower activity of cellulase to degrade same amount of biomass. However according to the Fig 3.9, highest sugars yield are from 180°C 10 minutes although biggest surface area is from 220°C 20 minutes treatment. This shows that although surface area is highest at 220°C 20 minutes, reduction in cellulose content (Table 3.3) will not allow further hydrolysis, yielding low sugars yield. The crystallinity index (Fig 3.7) which increased throughout the pretreatment condition also play a role in reducing the sugar's yield.

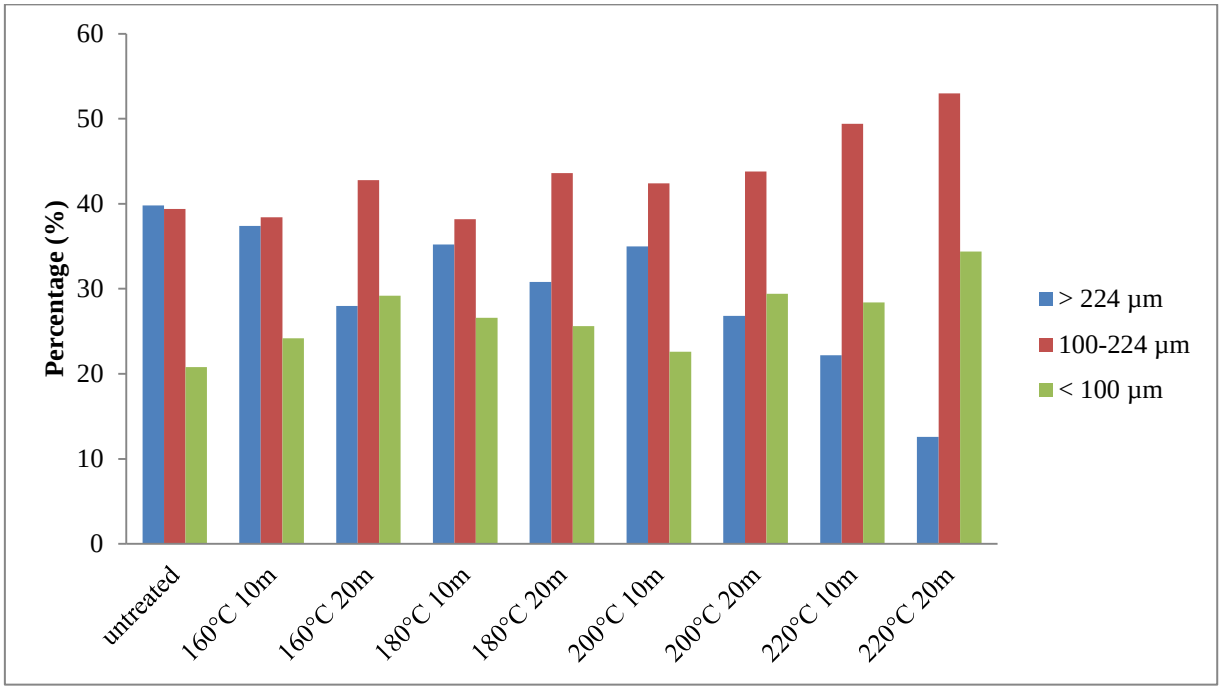


Fig 3.8 Particle size distribution of petiole residue after SHS treatment.

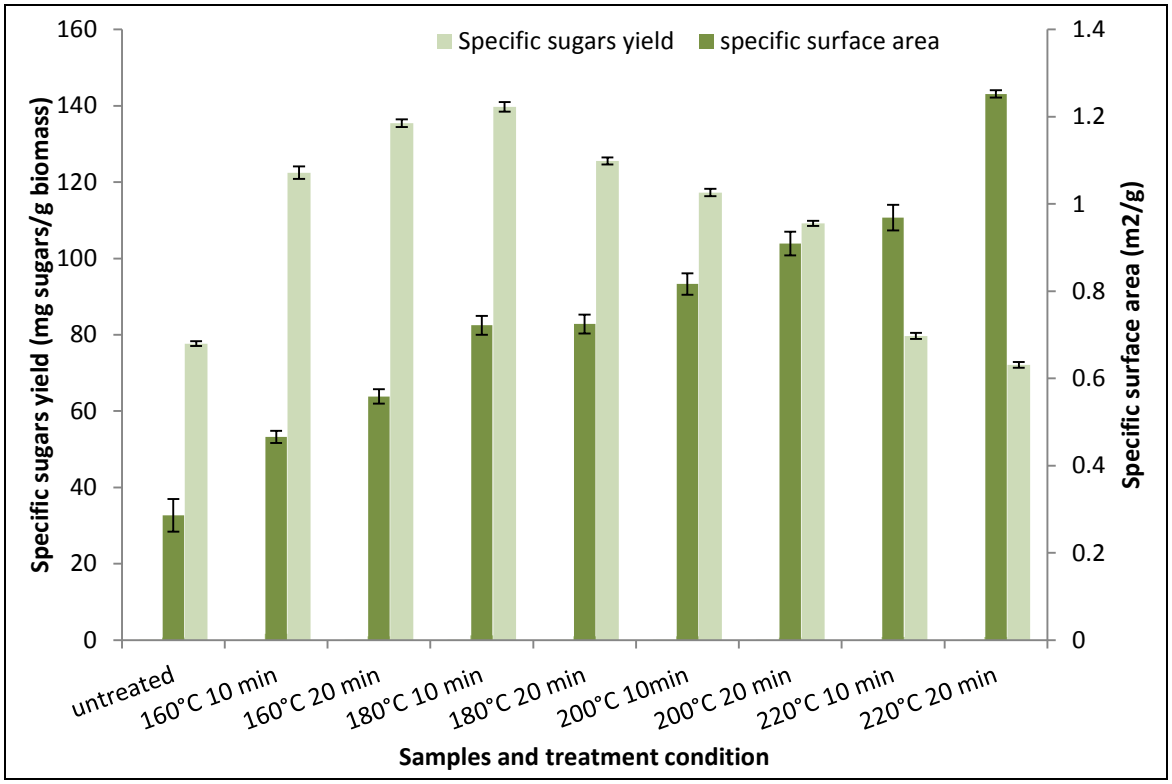


Fig 3.9 Specific surface area and specific sugars yield of petiole residue's sample after various SHS treatment's condition.

3.3.8 Comparison of SHS between OPF petiole residue, OPEFB and OPMF

One of the most notable finding in this study is that the shorter time treatment of petiole, as compared to longer duration for OPEFB and OPMF (Table 3.4). This might be due to the initial softer and porous fibre blocks that built petiole residue, as compared to strong and compact fibre strands that built OPEFB and OPMF (Bahrin *et al.*, 2012; Nordin *et al.*, 2013). Upon pressing for juice collection, petiole residue also becomes crushed, which opens the fibre internal components making it exposed for the SHS treatment. Moreover, SHS steams introduced to the fibre were at atmospheric pressure therefore it penetrates faster in porous fibre as compared to compact strands. That is why upon

exposure at long duration, petiole residue's fibre tends to disintegrate as it has lower thermal decomposition. However this is only applicable to freshly cut petiole, whereby old petiole usually become dried and shrink, making the fibre compact and difficult to degrade by SHS.

Additionally, both OPEFB and OPMF also contain some oil residue, which are still remains after oil collection process (Table 3.4). This palm oil residue have a specific heat of 2.358 J/g °C at 180°C, which hinders the full effect of SHS on OPEFB and OPMF as compared to dried petiole residue. This was proved during the material balance study of SHS effects on OPMF, whereby white coloured solid was found accumulated in the distiller unit (Fig 3.10). This white coloured unit was not found during the SHS of petiole residue. Hence it was extracted using hexane (since water could not dissolve it) and dried prior to analysis using FTIR.

Table 3.4 Comparison of SHS effects of petiole residue, OPEFB and OPMF.

Sample (Cellulose/ Hemicellulose/ Lignin) (%)	Highest cellulose concentration after SHS treatment	Suggested treatment condition[a]	Weight lost during treatment (%)	Source	Oil content
OPEFB (71.3/14.2)[b]	(not mentioned)	180°C 60 minutes	7.48	Bahrin *et al*., 2012	2.5% (Ellis and Paszner, 1994)
OPMF (42.81/33.10/20.49)	41.39	190°C 60 minutes	(not mentioned)	Nordin *et al*., 2013	8% (This study)
Petiole residue (46.02/38.54/15.44)	56.56	180°C 10 minutes	16.60	(This study)	0% (This study)

[a] Based on highest cellulose concentration or hydrolysis degree
[b] Bahrin *et al*., 2012 reported cellulose and hemicellulose combined concentration of 71.3%

Fig 3.10 Solidified palm oil at the distillation units.

Fig 3.11 shows the FTIR spectra of the white coloured solid which shows similar properties of a palmitic oil, or known as palm oil. Additionally, SHS treated OPMF shows changes in characteristics due to the removal of the palmitic oil

from its fibre. Based on calculation, the palm oil collected through the SHS process amounted 1.21% from the initial 8% remaining oil. This finding opens a new window of exploration for the residual oil collection from OPMF through the usage of wasted SHS, although it is important to further optimize the conditions in the future for a better yield of the oil. In parallel, water collected through the distillation process may be recycled for the use in the production of the SHS by the steam generators. This will reduce the water consumption of the palm oil mill, as well as reducing waste production.

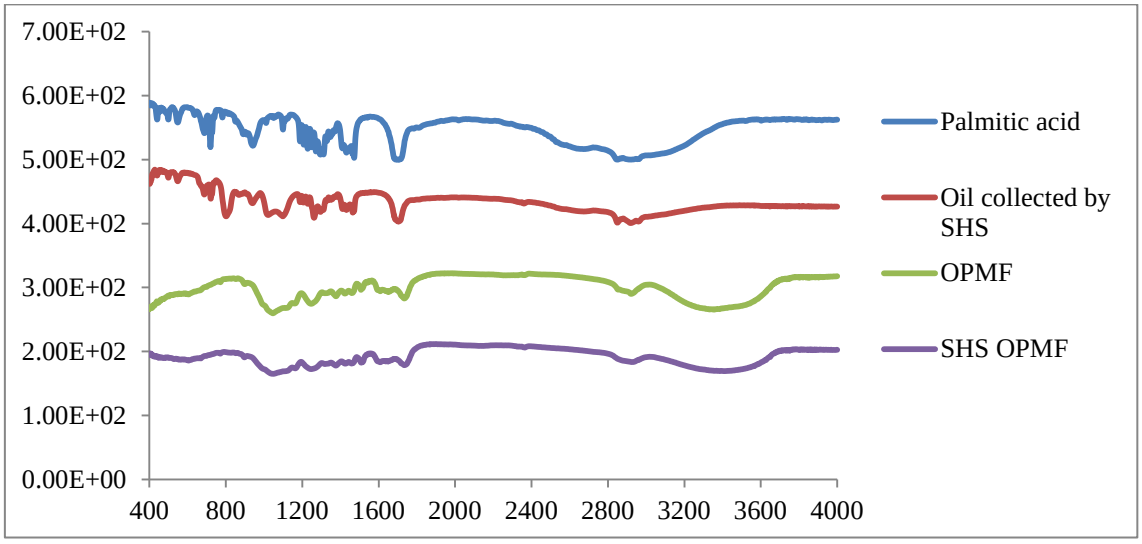


Fig 3.11 FTIR spectra of pure palmitic acid as compared to oil collected by SHS from OPMF. OPMF spectra prior to SHS and after SHS also show some changes due to the collection of the oil.

3.3.9 Performance of petiole residue saccharification after SHS pretreatment as compared to WDM pretreatment

WDM pretreatment was chosen as comparison due to its high effectiveness for enzymatic hydrolysis, without generating inhibitor for fermentation (Hideno *et*

al., 2009). WDM could also conserve all of the pretreatment's products in its solution without the need for chemical, as well as pH alteration. Table 3.5 shows comparison of sugars yield in 5, 10 and 20 WDM cycles, after saccharification of petiole residue using 10 FPU of cellulase. Specifically for non-thermal WDM petiole residue, the addition of WDM cycle will enhance glucose yield throughout all the range used. This reflects that the particle size of WDM petiole residue is getting finer throughout the range. Although the particle size could become even finer with higher WDM cycle and enhance the saccharification, it will increase production cost tremendously in industrial scale. Furthermore there will be an optimum number of cycles where further cycle will not enhance saccharification yield. Hence, it was suggested that the additional of thermal treatment onto WDM products could enhance the sugars yield at lower WDM cycle (Roslan *et al*., 2011).

Table 3.5 Sugars yield from WDM petiole residue after saccharification.

WDM Sample	Glucose yield (g glucose/g petiole residue)		
	0 h	24 h	48 h
5 cycles	0.028	0.102	0.133
10 cycles	0.024	0.115	0.162
20 cycles	0.025	0.162	0.198
5 cycles + thermal	0.026	0.166	0.182
10 cycles + thermal	0.029	0.219	0.222
20 cycles + thermal	0.027	0.233	0.234

The results of thermally treated WDM petiole biomass demonstrated prove of this concept. It can be clearly seen that WDM petiole residue with thermal

pretreatment are advantageous over non-thermally treated WDM petiole residue, with WDM 10 cycles with thermal is superior as compared to WDM 20 cycles. There is also no significance difference between thermally treated WDM of 10 and 20 cycles. It shows that the optimum number of cycles could be at 10 with thermal, whereby further cycles with thermal could produce just a small amount of increment, as demonstrate in the Table 3.5. Usage of WDM is advantageous because it will conserve everything which is trapped within the biomass, into the product, which is a good characteristics for the petiole residue since there are still some residual petiole juice remain after pressing. The residual petiole juice will contribute to additional sugars which was in free form and will be available for further use, such as for fermentation.

One of the important things to highlight here is the increment in yield of sugars after saccharification, over untreated petiole. Fig 3.12 shows this comparison whereby performance in saccharification of untreated petiole as compared to 10 cycles thermally treated WDM treated petiole residue. It can be seen that there is an increment of almost 100 g sugars / g biomass for both glucose and xylose. On the other hand, there is only a small amount of increment of fructose and sucrose as both actually originated from the free sugars in the petiole biomass.

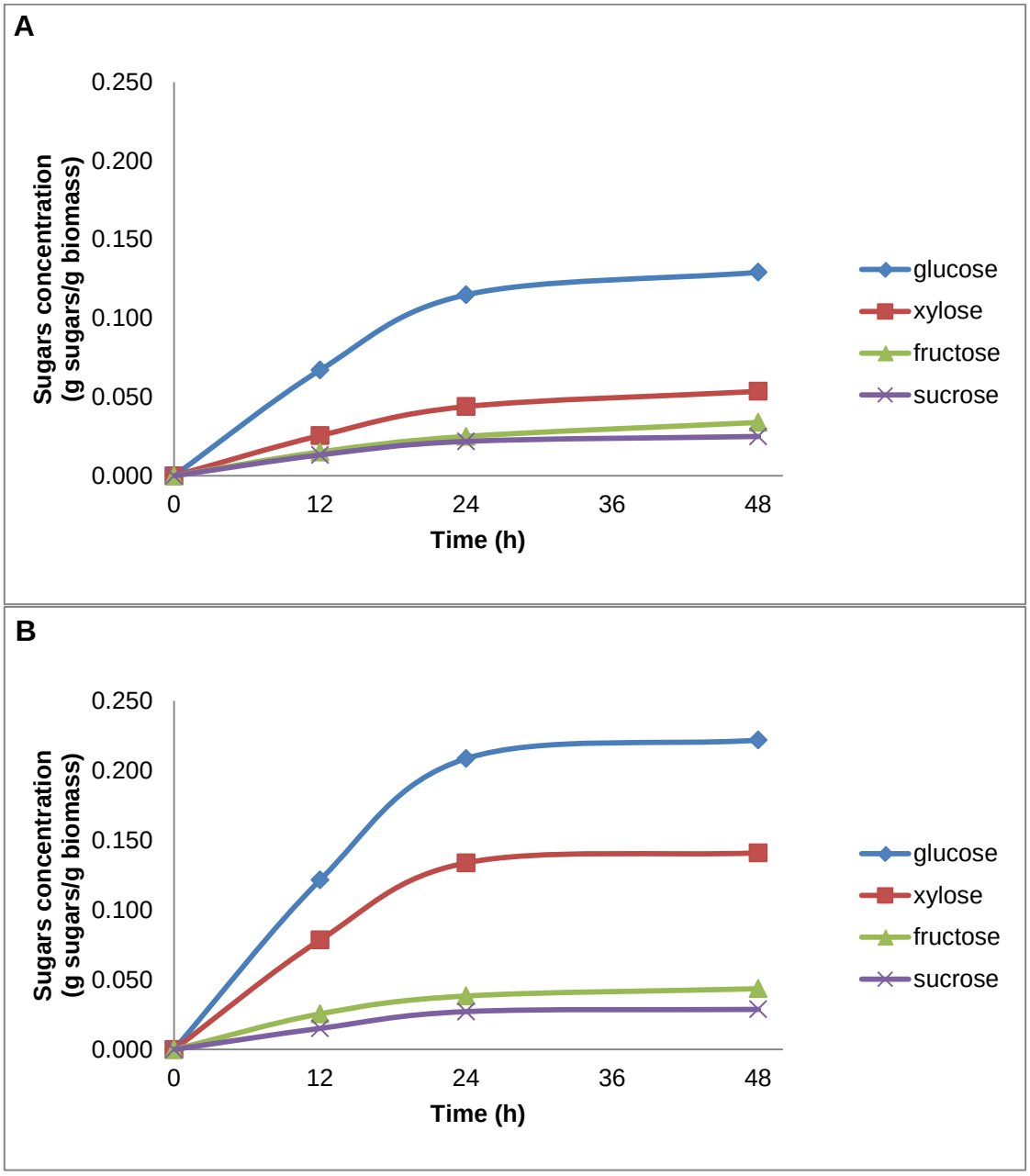


Fig 3.12 Comparison in saccharification performance between untreated petiole (A), with thermally treated WMD petiole residue (B).

By taking the best condition of SHS into account, which is 180°C for 10 minutes, and compare it directly with WDM with thermal treatment, it can be clearly observed that WDM is superior over SHS, in term of saccharification

performance. This is due to the fact that the WDM only grind, reduce the size, while at the same time conserve everything inside the biomass (Hideno *et al*., 2009). On the other hand, SHS pretreatment remove a portion of the biomass which is in the treatment temperature range, resulted in lower concentration of cellulose and hemicellulose, as well as removal of petiole juice. This can be observed in Table 3.2 where there are weight lost, as well as cellulose and hemicellulose. Removal of cellulose and hemicellulose is actually reducing the sugars precursor which finally reflected in lower sugars yield.

Furthermore WDM with thermal pretreatment perform better in saccharification due to contribution of a higher surface area as compared to SHS, which is 3.34 m^2/g compared to 0.722 m^2/g, respectively (Fig 3.13). Higher surface areas, which provide more contact for cellulase enzyme to take action, substantially improve the petiole residue digestibility by enzyme. This data is supported by the surface roughness analysis by using laser microscope, as in Fig 3.14. In this data, the surface of the SHS treated petiole (Fig 3.14B) is still rough and almost similar to untreated petiole residue (Fig 3.14A), with maximum height of 439.4 µm. On the other hand, WDM samples show a smooth surface with a maximum height of 236.1 µm (Fig 3.14C).

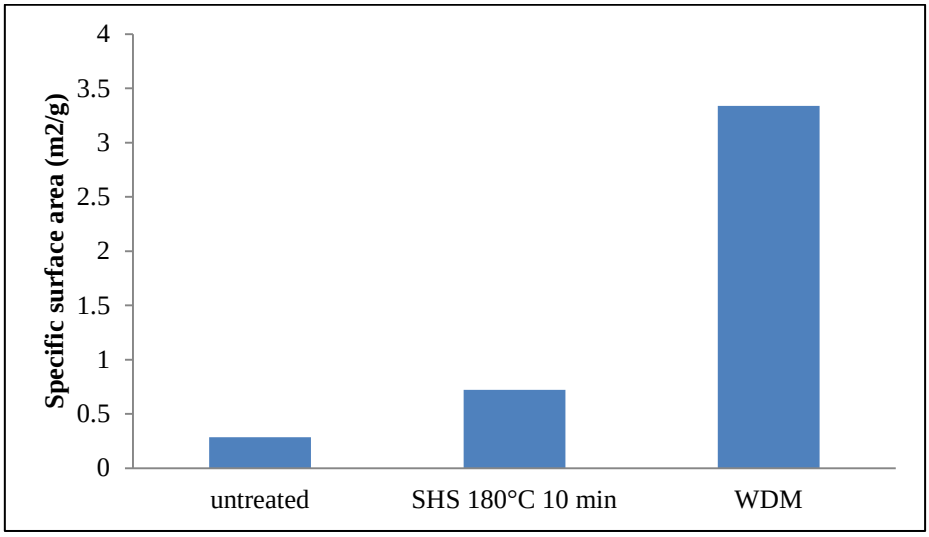


Fig 3.13 Specific surface area of untreated, SHS and WDM.

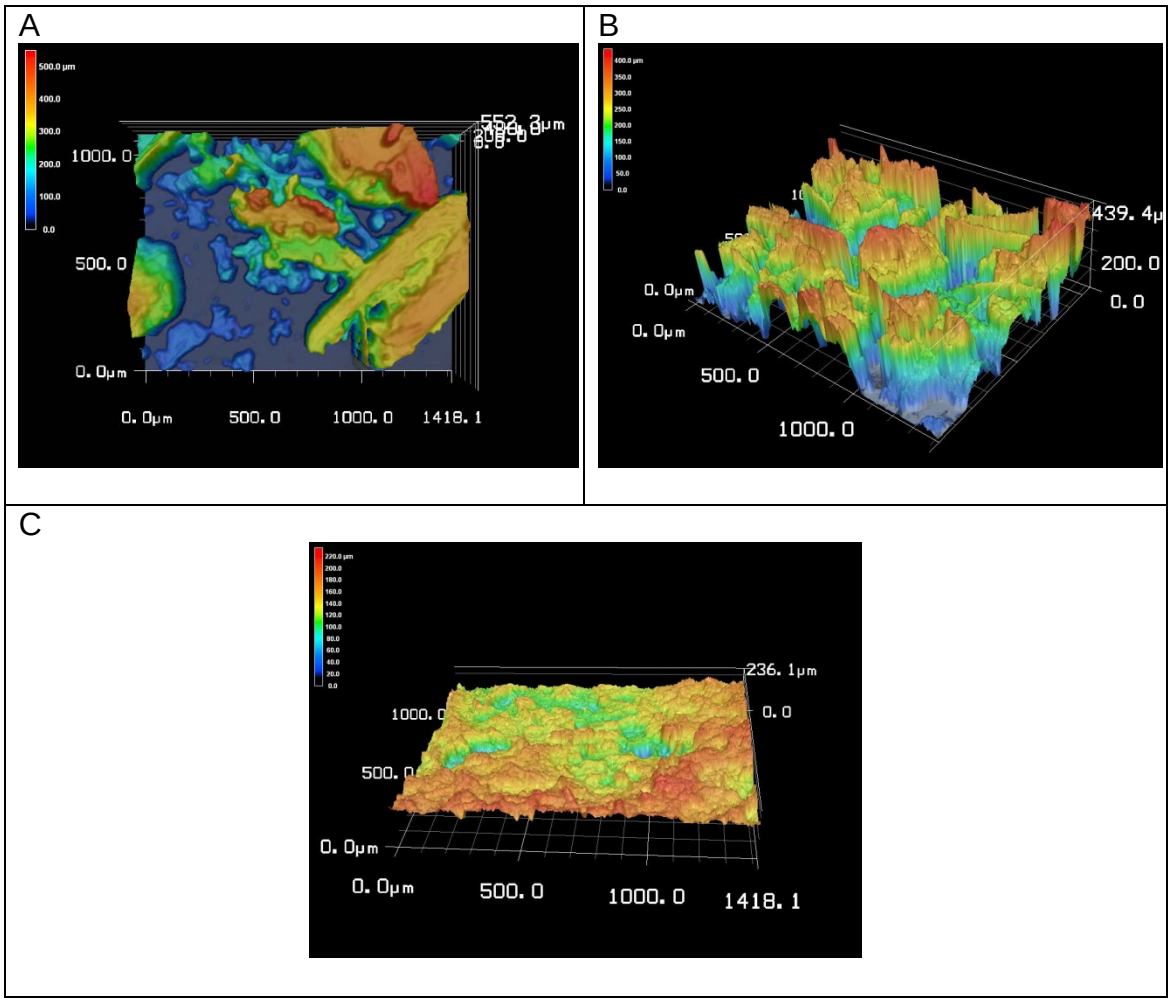


Fig 3.14 Roughness surface analysis of (A) untreated petiole residue, (B) SHS treated petiole residue, and (C) WDM treated petiole residue.

However, to prepare a disc milling device in palm oil mills is costly. This is due to the facts that the machine has to be built of stainless steel materials to avoid corrosion and reduce maintenance. Furthermore, the grinding stone used the milling process will has to be replaced after a certain amount of operation time due to the worn effect. As the process requires water in its medium, it is also important to incorporate additional water cooling system as the grinding stone tends to get very hot during the process, with risk to break. This will increase water consumption of the palm oil mill. Above all, power consumption of WDM is relatively high, which require additional energy to be supplied to the machine. WDM pretreatment is also time consuming, whereby in this study, it took about 60 minutes to complete a 10 cycle disc milling, and another 15 minutes for thermal treatment. Overall, to initiate and maintain the WDM process will involve high cost initially, and in the long run. These make the use of WDM in current practise as not practical, and could be low in profits.

On the other hand, SHS is excessively available in the palm oil mill, therefore the steam production is none or very minimal. Construction to be considered is only related to the piping of the steam from the exhaust pipe to a chamber, or conveyer belt, at which the petiole residue pretreatment take place. Maintenance of the machineries is minimal because the process will be dried all the times, with no physical contact such as in disc milling. Leaking of liquid can be omitted as the petiole residue will always remain in dried solid forms. Additional energy required is also minimal as compared to WDM, which is

mainly just to move the petiole residue inside the pretreatment chamber or conveyer belt. This is the reasons of why SHS if much favourable in the industry over SHS. Over times, it is predicted that the SHS system will profit the industry more as compared to WDM, although economic studies has to be performed to prove this concept.

3.3.10 General suggestion

From all of this information, it is safe to suggest that petiole residue conversion to biosugars is a novel way of generating more income to the mill. It can be done in the palm oil mill due to following facts: 1) utilization of petiole will not disturb the nutrient recycling in the plantation (Roslan *et al*., 2014); 2) petiole transportation to the mill is a matter of logistic which can utilize the same method for transportation of FFB, which is by the addition of another carriage behind the existing truck for FFB transportation; 3) SHS is being produced excessively in palm oil mill and the remaining potential energy in the steam is not being tapped, but is being released to the air; 4) short time exposure of petiole residue to SHS means that the process is not time consuming and should not use high amount of energy; and 5) palm oil mill may generate more income by selling SHS petiole residue for biosugar production, or selling biosugars and the sugar-rich juice pressed from the petiole.

Usage of other pretreatment methods such as chemical or WDM are welcome. But under current circumstances, profit margin could be jeopardized with the

use of expensive machineries such as a WDM, which also is high in maintenance, as well as the pretreatment is time consuming. In contrary, short time exposure of SHS at normal atmospheric pressure onto petiole residue, reflects to a simple device required for this pretreatment, for example a closed conveyer belt with retention time of 10 minutes. Petiole residue will be mobilized on the conveyer belt from point A to point B in a close system for 10 minutes, whereby the steam is being flowed through the system. This efficient system will produce a big amount of pretreated petiole residue in short time.

Use of concentrated cellulase, for example at 50 and 100 FPU, in a saccharification process will produce higher sugars concentration. However the cost of the cellulase used will increase 5 fold or 10 fold for the same amount of biomass used with lower efficiency in sugars production. Hence, lower concentration of cellulase, for example 10 FPU, can be used, to reduce the saccharification cost. Furthermore, SHS treated petiole residue is expected to be very high in volume with the application of short time pretreatment.

Given application of above technologies, the palm oil industry is at advantage to be promoted as green industry, which can overturn the negative global perception towards palm oil. Wasted steam generated from the mill not only can be used for pretreatment for biosugars production from petiole residue, but also for OPEFB, OPMF and others biomass to produce many other products. Additionally, if applied, this system will not disturb the nutrient balance in the oil

palm plantation, therefore it can be considered a win-win-win strategy for business, people and environment.

3.3.11 Other potentials of SHS and petiole residue

Isolation of lignin from petiole residue is also possible with the use of SHS. Since the hemicellulose and cellulose have lower thermal decomposition temperature which is around 160 – 340°C (Nordin *et al*., 2013), as compared to lignin which is around 200 - 500°C (Brebu and Vasile, 2009), SHS temperature and time can be set up to a higher rate to remove cellulose and hemicellulose. Experimental setup using the same equipment as in Fig 3.1B allows precise observation in material balance. Although current study is limited to the removal of hemicellulose only, further studies can be performed to fully accomplish this goal. However, total removal of hemicellulose alone is also enough as separation between lignin and cellulose is possible with the chemical pretreatment of sodium hydroxide (NaOH). Lignin, which will dissolve in the NaOH will be separated from the solid cellulose, at which both can be purified. This allows further use of lignin and cellulose, for example in the production of lignophenol, or nanocellulose (micro fibrillated cellulose, MFC) respectively.

Improving thermal stability of the biomass is also possible with the use of SHS, whereby optimized condition will result in stronger and higher crystallinity biomass, yet is very fine in size. This information is based on the crystallinity data (Fig 3.7), particle size distribution data (Fig 3.8) as well as specific surface

area data (Fig 3.9), at which higher temperature SHS treatment yield a smaller fibre particle, larger in surface area but with higher crystallinity. These combined characteristics contribute to the fibre's thermal and structural stability, which are important in the production of biocomposite to withstand sunny weather, high temperature and moist application, and may last over times (Muller *et al*., 2009).

In this study, it was also found that gross energy (GE) per gram of the SHS treated petiole residue increased towards higher temperature range and duration (Table 3.6). This shows that the petiole residue gain additional GE while becoming drier and sustaining weight lost. Increment of GE throughout the SHS pretreatment range, which also caused it to dry, suggest that usage of even higher SHS temperature could eventually make petiole biomass a suitable candidate for fire briquette, or biocharcoal, although further experiments has to be carried out to further explore this possibility. This suggestion is also made based on visual observation at which the petiole residue started to self-ignite after it was taken out of the SHS machine, and being introduced with external air.

Table 3.6 Comparison of petiole residue's gross energy after different condition of SHS pretreatment.

Sample	Percentage of weight lost (%)	Gross energy (cal/g)
untreated	0.00	4009.07
160°C 10m	15.70	4132.33
160°C 20m	17.00	4170.23
180°C 10m	16.60	4133.37
180°C 20m	18.20	4241.47
200°C 10m	17.50	4221.4
200°C 20m	19.60	4249.46
220°C 10m	22.50	4284.41
220°C 20m	26.10	4319.95

It was also suggested that cellulose remains after saccharification could also be of higher crystallinity. This is because lower concentration of cellulase activity may only hydrolyze lower crystallinity cellulose, leaving high crystallinity cellulose intact. This can be confirmed by using WAXD in the future, in which if it is confirmed, means that the remains of the higher crystallinity cellulose could also be converted into more sugars by applying second stage saccharification with higher cellulase activity, or with longer saccharification duration. The solid cellulose, as well as the hemicellulose and lignin, may also be separated from the hydrolysate, for further conversion into other suitable products such as biocomposite, as well as lower value products such as biocompost.

3.4 Conclusion

It is found that SHS at 180°C for 10 minutes is an efficient pretreatment of petiole residue for biosugars production, which is more practical and faster as

compared to WDM treatment method. Usage of 10 FPU on the petiole residue treated with SHS at 180°C for 10 minutes, demonstrates improvement in efficiency of specific sugars yield by 79.91% as compared to untreated. Ten FPU of cellulase concentration also shows a better efficiency in sugars yield as compared to 50 and 100 FPU in term of g sugars / g FPU used. Short duration of SHS pretreatment is achievable due to the fibre's morphological changes after pressing and grinding, as well as there is no remaining palm oil in the petiole residue, such as observed in OPEFB and OPMF. SHS also has advantages due to its short time pretreatment, as compared to WDM, which is time consuming, as well as require high maintenance.

In the next chapter, sugars yielded from the saccharification of SHS treated petiole residue will be subjected to bioethanol fermentation. In this fermentation, commercial nutrients will be replaced with petiole's juice, which also contain nutrients. It is hypothesized that the fermentation performance will be better or at par with the one that use commercial nutrients.

CHAPTER 4: BIOETHANOL PRODUCTION FROM OIL PALM FROND PETIOLE RESIDUE INDUCED BY NATURAL MICRONUTRIENT FROM PETIOLE'S JUICE

4.1 Introduction

After a suitable condition of SHS was found in the previous chapter, the sugars obtained from the saccharification were subjected to fermentation by Baker's yeast (*Saccharomyces cerevisiae*), to produce bioethanol. This process usually involves the addition of nitrogen source, as well as others minerals and vitamins to enhance the microbial activity in the fermentation process. In order to reduce the fermentation cost, utilization of cheaper substitutes to the commercial supplement (nutrients and minerals) must be used. In this study, fermentation was carried out with a modification, whereby the commonly used commercial supplement (Mandel's medium) was replaced with petiole's juice. This modification was made based on the finding that petiole's juice contains some of the nutrient required, including nitrogen, which is crucial to sustain the growth of microorganisms during fermentation (Medina *et al*., 2012; Zahari *et al*., 2012). By replacing the Mandel's medium with petiole's juice, further fermentation was performed without depending on the commercial nutrients to observe the effect of petiole's juice onto the bioethanol production rate by the yeast. Performance of the sugars fermentation with supplementation of petiole juice was compared

with sugars fermentation supplemented by Mandel's medium, as well supplemented by combination of both petiole's juice and Mandel's medium.

4.2 Materials and Methods

4.2.1 Media and yeast preparation

Mandel's medium was prepared according to Mandels (1974). One litre of Mandel's medium at pH 4.8 contained 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4.7H_2O$ and 1 ml of trace element. Mandel's medium were sterilized by autoclave at 121°C for 15 minutes, except for trace element which was filter sterile using 0.45 µm filter. It was then kept at 4°C until further used. Meanwhile petiole's juice was obtained by pressing of OPF petiole using Mini Mill (Matsuo Co. Ltd) (Fig 2.2) and sterilized by autoclaving at 121°C for 15 minutes. It was then kept at 4°C until further use.

Nutrient content of the petiole's juice is shown in Table 4.1, with initial glucose concentration of 42 g/L. Sugars for this fermentation was produced from 24 hour saccharification of petiole residue which was treated by SHS at 180°C for 10 minutes. Enzyme used during the saccharification is Acremonium cellulase with concentration of 10 FPU at 50°C. The product of the saccharification (hydrolysate) was then concentrated using rotary evaporator to remove up to 75% of the water content.

Table 4.1 Comparison of Mandel's medium and petiole's juice nutrients.

Mandel's medium		Petiole's Juice*	
$(NH_4)_2SO_4$	1.4 g/L	N	0.8%
KH_2PO_4	2 g/L	C	39%
$CaCl_2$	0.3g/L	C/N	50%
$MgSO_4.7H_2O$	0.3 g/L		
Trace element	1 ml/L	S	0.4%
		P	0.02%
		K	2.3%
		Ca	2.9%
		Mg	0.5%
		B	2 ppm
		Mn	2 ppm
		Cu	2 ppm
		Fe	66 ppm
		Zn	9 ppm

* Data was obtained from Zahari *et al*., 2012

S. cerevisiae were cultured on a potato dextrose agar (PDA) for 24 hour in incubator at 37°C prior to subculture into a yeast-peptone-D-glucose (YPD) broth for 24 hour on a shaker incubator at 37°C. It was then centrifuged at 500 ×g to separate the yeast cells and the supernatant. After discarding the supernatant, the yeast was then resuspend in sterile distilled water prior to inoculation into the fermentation medium.

4.2.2 Fermentation conditions

Fermentation was carried out in 250 ml conical flasks, by adding 50 ml of concentrated hydrolysate, 10 ml of either Mandel's media or petiole's juice, and 39 ml of distilled water, prior to inoculation of 1 ml of *S. cerevisiae*. Fermentation of combined Mandel's medium and petiole's juice supplementation (combined supplied medium, CSM) was performed by adding

5 ml Mandel's medium and 5 ml of petiole's juice, respectively. Fermentation was carried out at 37°C for 48 hour with sampling interval of 6 hour for the first 24 hour. All samples were centrifuged at 500 ×g to remove the pellet. The supernatants were then filtered by using 0.45 µm filter to remove suspended solids, prior to storage at -20°C.

4.2.3 Sugars and ethanol analysis

Sugars and ethanol concentration was measured using an autosampler, high performance liquid chromatography (HPLC, Shimadzu) with refractive index detector (RID) and KS-802 (Shodex) column. Mobile phase is sterile distilled water with flowrate of 0.6 ml/min and sample retention time is 20 minutes.

4.3 Results and Discussions

4.3.1 Reuse of petiole juice for the supplementation in fermentation

Petiole juice used in this study originated from the same source as the petiole residue. There is an option to use the petiole juice as the main carbon source, directly for fermentation but it requires the petiole juice to be concentrated (Zahari *et al*., 2014). However in this study, main carbon source will be originated from petiole residue, while petiole juice will become nutrients supply. This is based on a study (Zahari *et al*., 2012) as well as another parallel study in this research, which reveals that the petiole juice consist of enough nutrient such as nitrogen, phosphorus and potassium to support the growth of

microorganism such as yeast. Process flowchart (Fig 4.1) was also developed based on the study of which the petiole could yield highest sugars from saccharification, as well as the effect of petiole juice is found at optimum. Although a parallel study found that it is possible to use petiole juice without sterilization, petiole juice used in this study was autoclaved to reduce the risk of contamination, whereby it slightly reduce the concentration of sugars inside it. The objective of reusing the petiole juice is to eliminate the cost of adding commercial nutrients.

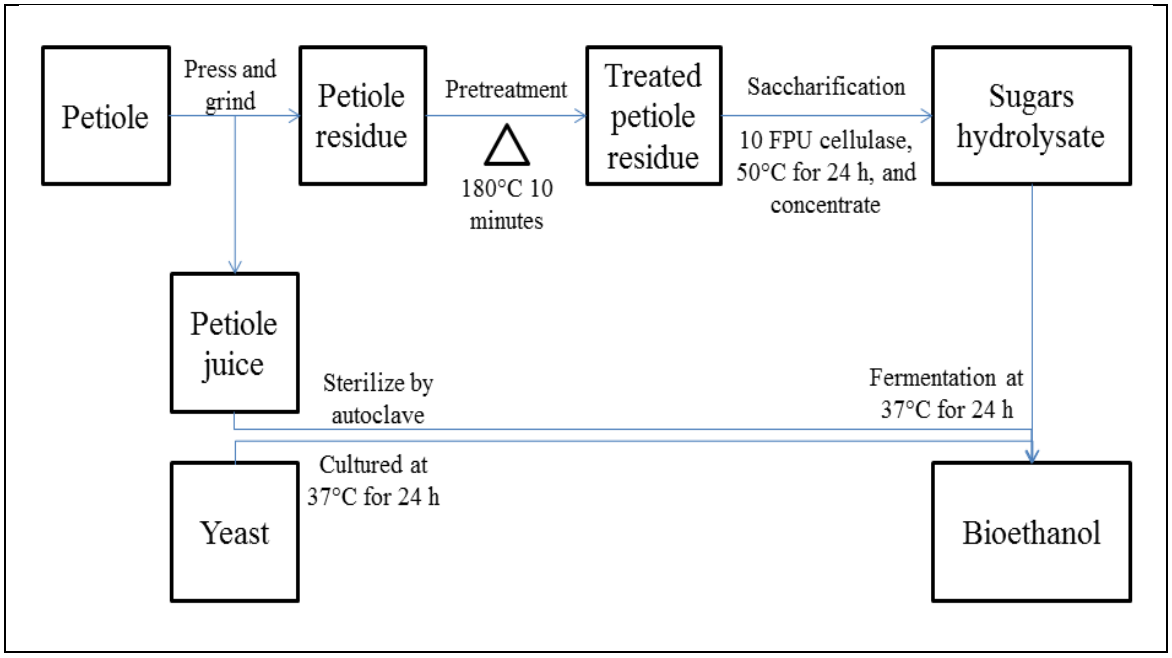


Fig 4.1 Processing condition applied in the fermentation with the supplementation of petiole's juice instead of commercial nutrients.

4.3.2 Concentration of sugars produced from saccharification

Saccharification of petiole residue in this study followed the optimum SHS condition found in the previous chapter, which is at 180°C for 10 minutes. The

sugars hydrolysate was then concentrated prior to fermentation to enhance the fermentation performance and bioethanol yield by *S. cerevisiae*. This concentration method is proposed because it can be easily achieved in the palm oil industry by using excess steam after the SHS pretreatment of the petiole residue. The steam which is now lower in temperature, may be exploited to evaporate water from the sugar hydrolysate to yield higher sugars concentration. The vaporized distilled water may then be recycled for the palm oil mill to utilize. In this study, glucose and xylose concentration prior to concentration process, and after concentration process were as in Table 4.2.

Table 4.2 Concentration of glucose and xylose, prior to water removal, and after water removal.

Sugars hydrolysate	Glucose (g/L)	Xylose
After saccharification	30.45	3.06
After removal of 75% water	111.8	11.75

4.3.3 Sugars consumption and bioethanol production

Fig 4.2 shows the glucose and xylose consumption as well as bioethanol production profiles, during the fermentation of sugars from saccharification of pretreated petiole residue. It can be observed that glucose was fully consumed during the first 24 hour for both Mandel's supplied medium and combined supplied medium (CSM), while xylose was fully utilized within 48 hour. On the other hand, petiole's juice supplied medium still contains a small fraction of glucose and xylose after 24 hour, by which are fully utilized after 48 hour. This

data reflects that the *S. cerevisiae* has no problem to consume both 6 carbon and 5 carbon sugars derived from the saccharification for bioethanol production, although it is mostly prefer 6 carbon sugars such as glucose.

Fermentations condition performed normally by using all 3 different supplement, Mandel's medium, petiole juice and CSM with bioethanol yield of more than 90% for each of supplement. The results also revealed that there is no significant difference in the fermentation performance, by replacing supplement of Mandel's medium with petiole's juice or with CSM (Fig 4.2).

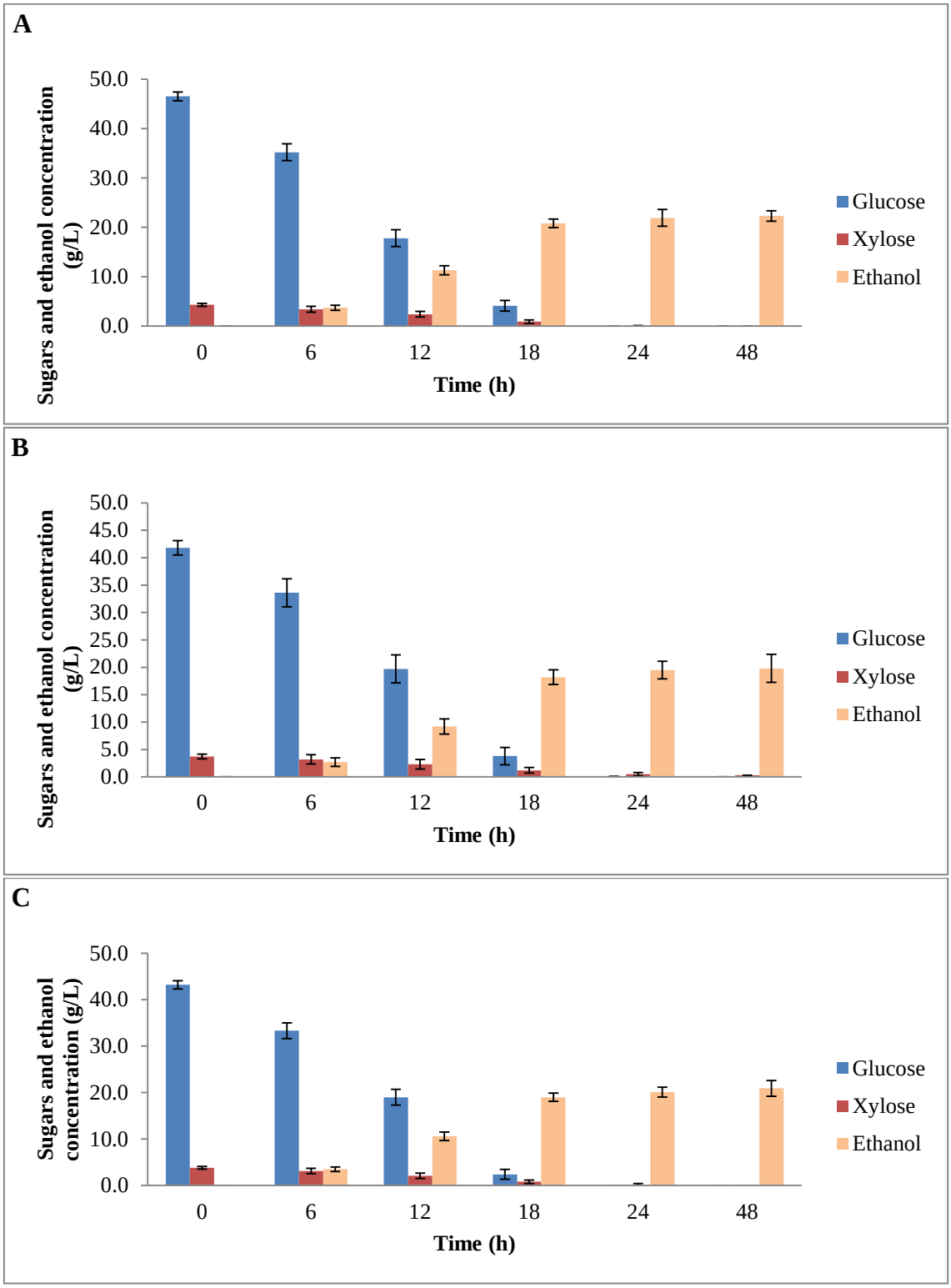


Fig 4.2 Sugars consumption and ethanol production in fermentation supplied with (A) Mandel's medium, (B) petiole's juice, and (C) combination of both Mandel's medium and petiole's juice, CSM.

These results also demonstrate the capability of petiole's juice to supply the highly demanding nutrient in any fermentation, which is the nitrogen. This is based on the fact that the fermentation carried out has no sluggish effect, as well as not a stuck fermentation. Studies reported that yeast fermentation for ethanol production is a nitrogen demanding process, whereby limitation in the nitrogen, as well as vitamin such as thiamine (Bataillon *et al*., 1996) and pantothenic acid (Wang *et al*., 2003), lead to a stuck or sluggish fermentation (Medina *et al*., 2012).

However in this study, replacement of 10 ml of Mandel's medium with the introduction of 10 ml of petiole juice with initial nitrogen concentration of 0.8%, into 100 ml fermentation medium, is capable to support microorganism's growth and fermentation throughout the fermentation duration of 48 hours. With all sugars as carbon sources in the medium were fully utilize within normal fermentation duration, and a yield of higher than 90%, it can be suggested that there is no inhibition effects by the petiole juice such as demonstrate by some other biomass for example paddy straw.

Inhibition of fermentation sometimes occurs due to the presence of aromatic compound, originated from the lignin. This inhibition cause the fermentation to become sluggish, with lower bioethanol yield, such as demonstrated by Roslan *et al*. (2009) using paddy straw. However, should the inhibition appears due to the aromatic compound, it may be easily solve by initially pretreats the biomass

using a mild sodium hydroxide (NaOH) treatment, to remove lignin, prior to saccharification.

4.3.4 Comparison of bioethanol production yield of petiole residue with OPEFB and OPT

Table 4.3 shows the comparison in bioethanol yield from yeast fermentation of others biomass originated from palm oil industry, such as OPT and OPEFB. From the table, it can be observed that the bioethanol yield is lowest from the OPT, followed by OPEFB and OPF petiole residue. As explained in chapter 3.3.8, the OPEFB is made of strong and dense fibre strands, which require extensive pretreatment to make it viable for the cellulase enzyme to biodegrade it. This lead to the need of higher concentration of cellulase during the saccharification process, as demonstrated by Jeon *et al*. (2014) in Table 4.3.

Table 4.3 Comparison of bioethanol yield from different biomass source, pretreatments, and cellulase activity used.

Sample	Pretreatment used	Cellulase activity	Microorganisms	Bioethanol yield	References
OPT	Aqueous ammonia	60 FPU / g glucan	*S. cerevisiae*	78.3%	Jung *et al*. (2011)
OPEFB	Changhae ethanol multiexplosion (CHEMEX)	40 FPU / g cellulose	*S. cerevisiae*	83.6%	Jeon *et al*. (2014)
OPF petiole residue	Superheated steam	10 FPU / g biomass*	*S. cerevisiae*	91.2%	(This study)

* Sugars hydrolysate was concentrated prior to yeast fermentation.

On the other hand, OPT which is higher in lignin as compared to OPEFB and OPF petiole (Table 2.1), require even higher concentration of cellulase for the saccharification. This is mainly due to the use of soft pretreatment condition (diluted ammonia) on the biomass. Higher lignin content will result in higher concentration of aromatic compound after pretreatment and saccharification. This aromatic compound is capable of slowing down the metabolism of *S. cerevisiae*, which is also the reason why the bioethanol yield is quite low.

Petiole residue is superior in this comparison due to an efficient pretreatment which successfully reduce the size of the biomass, as well as partially remove components that might inhibit the yeast performance (Bhuiyan and Hirai, 2000). Furthermore, the sugars hydrolysate after saccharification was concentrated, which enhance the fermentation ability of the yeast. Additional of petiole juice could also be supporting the yeast fermentation ability, which finally resulted in a higher bioethanol yield.

4.3.5 Additional value for the petiole juice

Findings from other studies revealed the use of petiole juice, without the addition of biomass for the production of P(3HB) as well as bioethanol (Zahari *et al*., 2012; Zahari *et al*., 2014). This shows that the petiole juice supplementation may not limited to bioethanol fermentation only, but also can be supplied into fermentation of others product, such as poly-3-hydroxybutanoic acids, biobuthanol as well as biovanilin. Although additional studies have to be

carried out in order to challenge this concept, it is highly suggested that petiole juice has the capability to perform as a substitute to commercial nutrients. If the petiole juice is made commercially available, for example in a spray dried packaging, it will substantially improve the value of the petiole juice, which will now be competing as commercial nutrients, and not only for sugars production.

4.4 Conclusion

It was concluded that fermentation of sugars from saccharified petiole residue can be carried out by replacing the commercial nutrients (Mandel's medium) with petiole juice. Partial substitution or total substitution of commercial nutrients with petiole juice will result in a similar performance in bioethanol fermentation. Petiole juice may also replace nutrients supplement for other fermentation. Life-sustaining nutrients found in petiole juice are capable of supporting yeast growth in the sugars medium and bio-converting it into bioethanol as the final products within 24 hours range, with yield higher than 90%. Fermentation of sugars derived from the biomass with supplementation of petiole's juice, are also free, or minimal in aromatic compound originated from lignin, which reflects to a high yield of bioethanol in a normal duration of fermentation which is 24 hours.

CHAPTER 5: CONCLUDING REMARKS AND SUGGESTIONS FOR FUTURE RESEARCH

Malaysia's economy is highly supported by the palm oil industry as its major commodity product in agriculture sector. As a strong and consistent industry, it has been consistently producing a lot of biomass through the processing, while at the same time, excessive energy are also being produced. Most of the time, this biomass and excess energy are being wasted due to its abundance. However, with the application of biotechnology and engineering, this wastage can be mitigated while generating additional income to the palm oil mill, creating additional jobs opportunities for peoples, as well as making the earth greener. This is the essence of the 3Ps – profit, peoples, and planet – to ensure the sustainability of the industry, people, and the earth.

From this study, it was found that the current good agricultural practise of leaving the OPF in the plantation for nutrient recycling has its significance. However, it was also found that the main contributors for the nutrient recycling are actually leaflet and rachis, with the structural support of stem. Petiole on the other hand, is rich in free-form liquid sugars and lignocellulose. It also has the least effect on the nutrient recycling as compared to leaflet and rachis, which means it is readily available for use. In addition, upon pressing of the liquid sugars, the petiole residue is physically pretreated. Since it still contain a

considerable liquid sugars trapped in the fibre, as well as lignocellulose which can be saccharified to produce even more biosugars, petiole residue offers big opportunity for new sugar line in the palm oil mill for additional income. Furthermore, transportation of the petiole is not as frequent as FFB, hence the transportation issue can be solve by adding extra carriage behind the existing FFB truck, when the petiole supply is enough. Therefore, it is highly suggested to take the petiole out of the plantation to make a better use of it.

It was also found that the excess superheated steam (SHS) generated by the oil palm mill is suitable to be used as the pretreatment of the petiole residue for biosugars production, which is at 180°C for 10 minutes. This condition of SHS yield 79.91% higher sugars yield in enzymatic hydrolysis as compared to untreated, although with higher concentration of cellulase enzyme, different results could be obtained. Although the use of wet disc milling (WDM) will yield a higher sugars yield, it is not practical for use in the palm oil industry as compared to SHS due to several reason such as high cost, maintenance issue and long pretreatment duration. SHS main advantage over any other physical and chemical pretreatment are process simplicity, as well as short time of pretreatment is enough to pretreat the petiole residue. This is mainly due to the structure of petiole residue which is more porous, softer, and not containing oil, as compared to OPEFB and OPMF. Additionally, pressing of petiole for juice collection already pretreats it, therefore it is much easier to be processed. However, the duration of SHS pretreatment may also be reduced to less than

10 minutes with higher temperature condition. This requires further studies in the future to significantly reduce processing time.

It was also estimated that about 50% of steam is being wasted from 165 900 – 240 900 tonnes of steam per year in a palm oil mill, hence in the future, it can be suggested to study how much of petiole residue can be pretreated by using this concept. Moreover, additional cost to introduce additional devices and equipment for the superheated steam process, including labour cost for the petiole handling must also be studied in the future to make sure that the business is reliable and sustainable. SHS condition can also be manipulated for other purpose such as for concentrating the sugars hydrolysate after saccharification, as well as for bioethanol distillation, which may further be studied in the future. Additionally, future studies can also be conducted to convert petiole residue into other products, climbing the value added chain, such as biofuel, biocompost, biocomposite and biochemical.

In a parallel study, it was found that petiole juice obtained by pressing of petiole contain nutrients which can support the growth of yeast for bioethanol fermentation. Fermentation carried out in this study revealed that the petiole juice is capable of replacing commercial nutrients in bioethanol fermentation with almost similar performance in bioethanol production yield to the commercial nutrients (Mandel's medium), petiole juice and combination of both. However, the cost for distillation to purify the bioethanol has not been taken into

account, hence further experiments must be conducted to study the cost of bioethanol fermentation in big scale in order to calculate if it is economically feasible for the industry to apply this technology for extra profit, as well as making bioethanol affordable as sustainable green fuel. It was also suggested to perform a study in the future, for the production of fermentation supplements, by using freeze-dried petiole juice.

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